Interleukin-6 Regulation of the Pancreatic Islet

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ABSTRACT (ENGLISH)

The fact that Interleukin-6 (IL-6) is systemically elevated in obesity and a predictive factor to develop type 2 diabetes provided the main rationale for the studies described in this thesis. The relevance and actions of IL-6 on the pancreatic islet are unclear. Thus, the overall aim was to investigate whether IL-6 plays a role in the regulation of pancreatic endocrine islet function and survival.

In the present thesis, the glucagon producing pancreatic α-cell is identified as a novel primary target of IL-6 actions. Furthermore, the pancreatic α-cell (specifically the glucagon granule) is a source of IL-6, and pancreatic islets release increased levels of IL-6 in response to a nutritional overload. Investigation of the tissue specific mRNA expression pattern of the IL-6 receptor demonstrated high expression of the IL-6 receptor in the endocrine pancreas, with highest expression on the α-cell. The islet IL-6 receptor is functionally coupled to STAT3, and IL-6 acutely regulates proglucagon mRNA and glucagon secretion, with no effect on acute insulin secretion. However, in support of previously published work chronic exposure to IL-6 impairs glucose stimulated insulin secretion, which is partly reversed by exogenous glucagon. Furthermore, IL-6 presents itself as a strong inducer of islet cell proliferation, with IL-6-induced α-cell proliferation occurring prior to β-cell proliferation. Intriguingly, IL-6 has distinct effects on α-cell and β-cell apoptosis. IL-6 protects α-cells from nutrient induced apoptosis, while it enhances β-cell apoptosis. *In vivo*, IL-6 knockout mice fed a short-term high fat diet reveal no metabolic phenotype, however, in contrast to wild type mice, the IL-6 knockout mice fail to expand α-cell mass in response to high fat diet feeding. After long term high fat diet IL-6 knockout mice without expansion of α-cell mass display decreased fasting glucagon levels. Despite this lack of α-cell mass expansion, high fat feeding of IL-6 knockout mice results in increased fed glycemia due to impaired insulin secretion, with unchanged insulin sensitivity, and similar body weights. Thus, IL-6 is necessary for the expansion of pancreatic α-cell mass in response to high fat diet feeding, and these data suggest that this expansion may be needed for functional β-cell compensation to increased metabolic demand.

The present work proposes that elevated IL-6 levels during obesity drive α-cell mass expansion and glucagon expression, which may be required for functional β-cell compensation in response to high fat diet induced insulin resistance. However, prolonged elevated IL-6 may lead to the observed pathological glucagon secretion at onset and during progression of diabetes.

ABSTRACT (GERMAN)

Das Zytokin Interleukin-6 (IL-6) ist in Individuen mit Übergewicht systemisch erhöht und ist ein prädikativer Faktor für die Entwicklung von Diabetes Typ 2. Der Effekt von IL-6 auf die pankreatische Insel ist unklar. Die Fragestellung der Studie war, ob IL-6 eine Rolle bei der Regulierung des endokrinen Pankreas spielt.

In dieser These wird die Glukagon produzierende Alphazelle als ein neues Ziel für den IL-6-Effekt identifiziert. Weiter wird gezeigt, dass IL-6 im Glukagongranulat nachgewiesen werden kann und bei einem Überangebot an Nährstoffen sezerniert wird. Untersuchungen von gewebsspezifischen RNA-Expressionsmustern haben gezeigt, dass der IL-6-Rezeptor im endokrinen Pankreas vor allem in den Alphazellen stark exprimiert wird. Der IL-6-Rezeptor in der pankreatischen Insel ist funktionell, kann STAT3 sowie die Produktion von pro-Glukagon mRNA und die Sekretion von Glukagon aktivieren ohne dabei einen Effekt auf die Insulinsekretion zu haben. Chronische Exposition von IL-6 vermindert jedoch die Glukose stimulierte Insulinsekretion, was mit exogenem Glukagon teilweise verhindert werden kann. Weiter zeigt sich, dass IL-6 die Inselzellproliferation stark fördern kann, wobei IL-6 zuerst die Alphazellproliferation und dann die Betazellproliferation induziert. Interessanterweise hat IL-6 einen anderen Effekt auf die Alpha- und Betazell-Apoptose. Interleukin-6 schützt die Alphazelle vor Nährstoff-induzierter Apoptose, erhöht jedoch die Betazell-Apoptose. *In vivo* IL-6 defiziente Mäuse haben bei kurzfristiger Haltung mit einer hochkalorischen Diät einen normalen Phänotyp und ihre Alphazellenmasse erhöht sich nicht. Nach längerer Haltung mit einer hochkalorischen Diät zeigen die IL-6 defizienten Mäuse auch eine Abnahme des Nüchtern-Glukagons ohne Erhöhung der Alphazellmasse. Trotz fehlender Zunahme der Alphazellmasse zeigen die Mäuse nach Fütterung eine erhöhte Glykämie, die auf eine gestörte Insulinsekretion zurückzuführen ist. Dabei ist die Insulinsensitivität und das Körpergewicht vergleichbar mit dem Wild-Typ. Interleukin-6 erhöht die Alphazellmasse, was für eine normale Betazellfunktion bei erhöhtem Insulinbedarf zur Kompensation nötig ist.

Aufgrund dieser Arbeit postulieren wir, dass das erhöhte IL-6, das man bei Übergewicht beobachtet, die Zunahme der Alphazellmasse kontrolliert und dass dessen Glukagon-Produktion für die Betazellfunktion zur Kompensation bei einer hohen kalorischen Diät nötig ist. Chronisch erhöhte IL-6-Werte könnten die beobachtete pathologische Glukagonsekretion bei Diabetes erklären.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

The maintenance of glucose homeostasis is achieved via an intricate network of regulatory pathways, and an elaborate interaction among several organs and hormones. The anabolic hormone insulin and its catabolic counterpart glucagon are the foremost hormones involved in this regulation. Dysregulated secretion of these pancreatic islet hormones, and an impairment in their biological actions results in hyperclycemia, a hallmark of Diabetes Mellitus type 1 and 2 (Diabetes, 2006). Whereas type 1 diabetes mellitus (T1DM) is caused by an immune-mediated selective destruction of the insulin producing β-cells (Atkinson and Maclaren, 1994), the immune system has classically not been associated with type 2 diabetes mellitus (T2DM). However, recently low-grade systemic inflammation has been identified as a hallmark of T2DM, and in those at increased risk of developing the disease (Kolb and Mandrup-Poulsen, 2005; Pickup, 2004; Wellen and Hotamisligil, 2005). Furthermore, islet associated macrophages are increased in T2DM (Ehses et al., 2007), thus associating activation of the innate immune system with type 2 diabetes. In line with this, interleukin-1 (IL-1) antagonism in patients with T2DM improves glycemia by improving pancreatic β-cell function, while concomitantly strongly reducing circulating interleukin-6 (IL-6) (Ehses et al., 2007; Larsen et al., 2007). Interleukin-6 is among the systemic indicators of inflammation reported to be highly associated with the disease. Circulating IL-6 levels are increased in obese individuals and patients with T2DM, and predict the development of T2DM (Herder et al., 2005; Spranger et al., 2003). Moreover, IL-6 receptor polymorphisms map to a region of repeated linkage to type 2 diabetes (Hamid et al., 2004; Wang et al., 2005). While much research has focussed on the role of IL-6 in the regulation of insulin sensitive tissues, the role of IL-6 in the regulation of the pancreatic islet is unclear.

1.2 THE DISCOVERY OF THE PANCREATIC ISLET HORMONES INSULIN AND GLUCAGON

1.2.1 The discovery of insulin

Prior to January 11th 1922 there was no treatment available for people with diabetes. The lack of treatment, leading to the death of many people, was a consequence of not having identified the substance that would relieve glycosuria (sugar in the urine). Already in the 19th century, a relationship between the pancreas and diabetes was demonstrated. Oskar Minkowski and Joseph von Mering from the University of Strasbourg removed the pancreas from a dog, and several days after having removed the pancreas, they found sugar in the dog's urine, and concluded that the dog had become diabetic. Diabetic research continued into the early 1900's, and at the turn of the century Eugene Opie from Johns Hopkins University discovered that diabetes is caused by something contained within the islets

of Langerhans, discovered by Hans Langerhans in 1869. Frederick Banting, a graduate of the University of Toronto Medical School, became interested in pancreas and diabetes research in 1920 and approached Professor John Macleod, a well known researcher in the field of carbohydrate metabolism. Along with Charles Best, a fourth year medical student at the time, and J.P. Collip, a well known biochemist, these four scientists discovered a substance that when injected into a pancreatectomized diabetic dog, abated the symptoms of glycosuria. This discovery was presented the same year in a paper that called this substance insulin. On January 11th, 1922, Leonard Thomson, a 14 year old diabetic who lay dying at the Toronto General Hospital, was given the first injection of insulin completely eliminating the symptoms of glycosuria. In 1923, Banting and Macleod were awarded the Nobel Prize for their discovery of insulin. Banting shared his award with Best, and Macleod shared his award with Collip.

The primary structure of insulin was determined in 1953 by Frederick Sanger (Sanger and Thompson, 1953a; Sanger and Thompson, 1953b), this was the first time a complete amino acid sequence of a protein was determined. Theoretically, this discovery allowed for the biosynthetic production of insulin. However, it was only in 1978 that human insulin was successfully produced using recombinant DNA technology and the extraction and purification of insulin from animals could be left aside. Today, 230 million people are diagnosed with diabetes worldwide (World Health Organization/International Diabetes Foundation), a number that is expected to increase to 300 million by 2025 (Zimmet et al., 2001). Alongside with the ultimate goal of finding a cure for the disease, the challenges today range from lack of diagnosis and availability of insulin in some parts of the world, to improving glycemic control, and replacing daily insulin injections by less invasive means.

1.2.2 The insulin gene and biosynthesis

The insulin gene was among the first genes to be sequenced. Its structure is highly conserved among species, and consists of three exons and two introns. Exon 1 is located in the 5' untranslated region of the gene. Exon 2 contains sequences encoding the signal peptide, the insulin B chain and part of the C-peptide, while exon 3 encodes the remainder of the C-peptide, the insulin A chain and the 3' untranslated region sequences (Bell et al., 1980). Most species express only one gene, however, in the mouse and rat, two non-allelic insulin genes exist, and these are equally expressed in islets (Giddings and Carnaghi, 1988). The single copy human insulin gene is located on the short arm of chromosome 11 (Harper et al., 1981; Owerbach et al., 1981). β-Cell specific expression of the insulin gene is dependent on DNA sequences located within a region upstream of the transcription start site (German et al., 1995; Philippe, 1991; Stein, 1993). The rat insulin promoter (RIP) I and II regions are highly conserved among species, and contain numerous regulatory elements interacting with β-cell specific transcription factors (reviewed by Melloul et al., 2002). Numerous transcription factors have been implicated in the expression of the insulin gene in pancreatic β-cells, Pancreatic Duodenum Homeobox-1 (Pdx-1), the basic helix-loop-helix transcription factor BETA2/NeuroD, and the Paired

box (Pax)-6 are among the key transcription factors regulating insulin transcription in the β-cell (Melloul et al., 2002). In addition to expression in the β-cell, insulin is expressed in the endocrine cells in the pituitary gland (Hrytsenko et al., 2007).

The insulin transcript is translated into a single 110 amino acid precursor, preproinsulin. Preproinsulin contains a hydrophobic 24 amino acid signal peptide, which when removed, gives rise to proinsulin, a 9000 kDa polypeptide chain that includes the insulin B and A chains (Chance et al., 1968; Steiner and Oyer, 1967). During the intracellular maturation of secretory granules, proinsulin is cleaved by prohormone convertase (PC)2 and PC1/3 to yield insulin and a 31 residue connecting C-peptide fragment. The cleavage site for PC1/3 is at the junction between the B chain and the C-peptide, this cleavage precedes the cleavage by PC2 at the C-peptide – A chain junction. The very stable ~5800 kDa globular insulin protein thus consists of the two peptide chains A and B linked together with disulfide bonds.

1.2.3 The discovery of glucagon

The history of glucagon begins with that of insulin. In 1921, when the team of Frederick Banting at the University of Toronto tested their first pancreatic extracts in pancreatectomized dogs, they observed that insulin-induced hypoglycemia was preceded by a transient, rather mild hyperglycemia. However, the discovery of glucagon must be credited to Murlin et al., in 1923 they suggested that the early hyperglycaemic effect of the pancreatic extracts was due to a contaminant with glucogenic properties, which they proposed to call glucagon, or the mobilizer of glucose (Seige, 1986). The pancreatic islet α-cell was found to be the source of glucagon in 1948 by Sutherland and DeDuve (Holst, 1983).

The primary structure of glucagon was described in 1957 (Bromer et al., 1957), and the description of a glucagon radioimmunoassay (RIA) by Unger et al. made it possible to investigate the physiology of glucagon and its role in various diseases. The first ever published paper evaluating the hormonal status of glucagon was published in 1962 (Unger et al., 1962). This paper provided specific identification of endogenous glucagon in plasma, and demonstrated the influence of blood glucose concentration upon glucagon secretion.

Since the initial description of the hormonal status of glucagon, a role for the α-cell and glucagon in the pathophysiology of diabetes has been demonstrated (see Chapter 1.7.2: Type 2 diabetes). Hyperglucagonemia contributes substantially to hyperglycemia in patients with diabetes, rationalizing glucagon antagonism as one of the most attractive targets for diabetes intervention.

1.2.4 The glucagon gene and its products

Glucagon is a member of the pituitary adenylate cyclase activating polypeptide (PACAP) superfamily of peptide hormones, which to date includes 6 genes encoding 9 bioactive peptides

(reviewed by Sherwood et al., 2000). These hormones are classified within this family based on their considerable sequence homology.

The proglucagon gene encoding glucagon, glucagon-like peptide (GLP)-1 and GLP-2 is expressed as a 2-kb transcript, and is located on the long arm of chromosome 2 (Schroeder et al., 1984; Tricoli et al., 1984). The proglucagon gene consists of a 5'-untranslated region, the protein coding region comprised of the N-terminal signal sequence, the proglucagon consisting of the GRPP (glicentin-related pancreatic peptide) and followed in order by the sequences that encode glucagon, GLP-1, and GLP-2 (Jin and Drucker, 1995). The glucagon, GLP-1 and GLP-2 sequences are interrupted by short spacer sequences that encode intervening peptides (IP)-1 and 2 (Kieffer and Habener, 1999). Five important transcriptional DNA control elements have been identified in the 2.5-kb of DNA sequence that 5' flanks the initiation of transcription of the rat proglucagon gene. The five DNA control sequences of approximately 20 - 40bp have been designated G1, G2, G3, G4, CRE (cyclic AMP response element), and ISE (intestine-specific enhancer) of GUE (glucagon upstream enhancer (Jin and Drucker, 1995; Philippe et al., 1988). The G1 element confers α-cell specific expression of the glucagon gene in the pancreas, the G2 and G3 elements are enhancers specific to islet cells (Philippe et al., 1988), the CRE allows for cAMP to regulate the transcription of the proglucagon gene (Knepel et al., 1990), and ISE is a determinant for the transcriptional expression of the gene in intestinal L cells (Philippe et al., 1995).

There are four known sites of expression of the proglucagon gene: the pancreatic α-cells, the L cells predominantly located in the distal ileum, colon, and rectum, and the nucleus tractus solitarius in the hindbrain, and in magnacellular neurons of the hypothalamus (Bernstein, 1984; Kieffer and Habener, 1999).

In mammals, the tissue specific expression pattern of the proglucagon gene is accomplished at the level of alternative posttranslational processing of proglucagon (Drucker et al., 1986; Mojsov et al., 1986; Novak et al., 1987). The processing of glucagon is remarkably specific; the predominant bioactive peptide produced in the pancreatic α-cell is glucagon, whereas in the intestine and brain the bioactive products produced are predominantly GLP-1 and GLP-2. Processing by PC1/3 results in the formation of GLP-1 and GLP-2. Prohormone convertase 2 contributes to processing proglucagon in the pancreatic α-cell to produce glucagon, a single chain 29 amino acid polypeptide with a molecular weight of 3483 kDa (Rothenberg et al., 1995; Rouille et al., 1995; Rouille et al., 1994).

1.3 THE PANCREATIC ISLET AND ITS HORMONES

1.3.1 Pancreatic islet architecture and composition

The pancreatic islets are scattered throughout the pancreas and constitute about 1 - 2% of the organ. They range in size from a few islet cells to several thousand islet cells per islet (Bonner-Weir, 1991). The pancreatic islet is classically described to contain four different endocrine cell types:

insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP-cells (Bonner-Weir, 1991). However, a fifth endocrine cell, the ghrelin-producing ε-cell was recently discovered (Heller et al., 2005; Prado et al., 2004; Wierup et al., 2004). Numerous ε-cells are present in islets during pancreas development and at birth, however, the population declines postnatally (Heller et al., 2005). In addition to endocrine cells, islets contain endothelial cells, nerves, fibroblasts, and extracellular matrix (Powers, 2008).

Pancreatic islet cell composition differs greatly between human and rodent islets. The relative proportion of β-cells in human islets is less than in rodent islets, 55% versus 77%, whereas the fraction of α-cells is greater in human islets than in rodent islets, 35% versus 18% (Brissova et al., 2005). Moreover, islet architecture is different, while normal adult rodent islets have a β-cell core and a non β-cell mantle, human islets do not have this core-mantle architecture, resulting in human β-cells being intermingled with other endocrine cells (Cabrera et al., 2006). Whether the islet architecture and/or endocrine cell composition have measurable consequences for islet function has not been thoroughly investigated. Nevertheless, a discrepancy in β-cell oscillation pattern in human and rodent islets has been reported to be a consequence of islet architecture (Cabrera et al., 2006). Rodent islets, with a β-cell core, display synchronized oscillations in membrane potential and intracellular Ca^{2+} in response to high glucose. This unique pattern is not observed in human islets, suggesting that human β-cells are functionally segregated, in line with the anatomical findings showing an absence of clustering.

1.3.2 Pancreatic islet blood flow

The pancreatic islets are highly vascularized, with at least one arteriole supplying every islet (Murakami et al., 1993). Compared to the exocrine portion of the pancreas, the islets receive a disproportionately large fraction (5 - 10%) of the total pancreatic blood flow (Lifson et al., 1985). The direction of blood flow, and the microvascular organisation have physiological significance, as hormones secreted from one endocrine cell type may act on downstream cells in a paracrine manner contributing to the communication between cells within an islet.

Morphological work performed in rodents has revealed an elaborate organization of blood supply, which led to the notion that blood flows in the direction of BAD (β-to-α-to-δ-cells) (Menger et al., 1994). Under these circumstances the arterial blood first reaches the β-cell core, and subsequently flows back to the islet surface comprised of α-cells and δ-cells (Bonner-Weir, 1988). The implication of the observed vascular arrangement is that β-cells, which are stimulated by glucagon and inhibited by somatostatin, may respond to circulating, but not locally secreted, glucagon and somatostatin. Superficial α-cells and δ-cells, on the other hand, may respond to locally secreted insulin, which reaches them either by intra-islet capillary circulation or by diffusion (paracrine effect). Unpublished work (however presented at the Keystone Symposia, Snowbird, April 2008 by Dr. Alvin Powers) suggested that islet blood flow is not one entity, the data presented revealed that there were differences

in blood flow among islets. In 2/3 of the islets, blood flow reached the β-cell core first then the α-cell/δ-cell mantle, whereas in 1/3 of the islet, blood flow reached the core and the mantle at the same time. In these islets, the arterial blood that reached the mantle flowed in a circular pattern on the edge of the islet, thus only reaching the cells in the mantle. Importantly, it was emphasized by Powers that they observed no islets where blood flow reached the α-cells first.

However, realising the difference in islet architecture and cell composition between rodent and human islets, it is not surprising that there is no evidence for such a vascular arrangement in human islets (Cabrera et al., 2006). The cell to blood vessel association in human islets is found to be random, and there exists no anatomical indication for an order in paracrine signalling as determined by the direction of blood flow. A proposed model in which any given endocrine cell can influence all other endocrine cells supports these observations (Brunicardi et al., 1996).

1.3.3 β-Cell insulin secretion

Circulating insulin secreted from the pancreatic β-cells is tightly regulated by blood glucose. The β-cell responds to blood glucose fluctuations by sensing concentrations of glucose. Glucose equilibrates across the plasma membrane via the glucose transporter (GLUT)2 and is phosphorylated by glucokinase, the rate-limiting enzyme of glycolysis. Pyruvate, the main product of glycolysis in the β-cell, enters the mitochondrial TCA cycle and provides the link between oxidative metabolism and insulin secretion. The ensuing increase in cellular ATP:ADP is due to electron transfer from the TCA cycle (in the form of NADH and $FADH_2$) to the respiratory chain (oxidative phosphorylation). Thereafter, membrane depolarization is facilitated through closure of ATP-sensitive K^+ channels (K^+_{ATP}), resulting in Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, and culminating in insulin granule exocytosis. However, this model is simplified and there exists a role for K^+_{ATP}-independent glucose-stimulated insulin secretion, Ca^{2+}-independent insulin secretion, and additive signals regulating insulin granule exocytosis (glutamate, malonyl-CoA) (reviewed in (Maechler and Wollheim, 2001).

Although glucose and an increase in intracellular Ca^{2+} concentration are the primary signals in the regulation of insulin secretion, other intracellular signals are also important; adenosine 3´,5´-cyclic monophosphate (cAMP) being especially critical. Glucagon, and the incretins GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are known to potentiate glucose-induced insulin secretion via cAMP signalling**.** The latter two hormones account for 50% of the insulin response following a meal, the so-called “incretin effect” (Creutzfeldt, 2005). Glucagon and incretin receptors are coupled to G proteins that activate adenylate cyclase, increasing cAMP generation. Until recently, the action of cAMP on insulin secretion was thought to be mediated exclusively by the activation of protein kinase A (PKA). Activated PKA can influence insulin secretion through effects on the ATP-dependent K+ channel, voltage-gated Ca^{2+} channels, on Ca^{2+} released from intracellular stores, and mainly on the process of exocytosis. However, accumulating evidence indicates that the cAMP-binding protein,

cAMP regulated guanine nucleotide exchange factor (cAMP-GEF)/exchange proteins activated directly by cAMP (Epac), participates in a novel PKA-independent mechanism of cAMP-stimulated insulin secretion. cAMP compartmentalization in pancreatic β-cells has been proposed to account for these distinct effects of receptor coupled cAMP signalling on insulin secretion.

1.3.4 α-Cell glucagon secretion

As the counter-regulatory hormone to insulin, glucagon secretion is suppressed in response to glucose, and increased when glucose levels are low. However, contrary to glucose-stimulated insulin secretion from the β-cell, the mechanisms underlying the regulation of glucagon secretion by glucose are not as well understood. It is not yet clear whether α-cells directly sense and respond to fluctuations in plasma glucose, or whether the response to low circulating glucose is mediated by the autonomic nervous system and/or the paracrine/endocrine effects of secretory products from other islet endocrine cell types. Currently, a large body of research favours the latter paracrine/endocrine hypothesis. Although neuronal modulation of α-cell activity is certainly operative, this is probably secondary to the inhibitory effects of β-cell secretory products during hyperglycemia (Kurose et al., 1992).

Studies on purified fractions of rat α-cells have revealed that they are regulated by glucose metabolism, they express the glucose sensor enzyme glucokinase (Heimberg et al., 1996) and depend on the low K_m GLUT1 for transmembrane glucose transport rather than GLUT2 as in β-cells (Heimberg et al., 1995). It is also well established that α-cells are electrically excitable and, unlike β-cells, generate spontaneous action potentials in the absence of glucose (Yoshimoto et al., 1999). Studies in guinea-pig (Rorsman, 1988; Rorsman and Hellman, 1988), mouse (Barg et al., 2000) and rat α-cells (Gromada et al., 1997) indicate that α-cells are equipped with voltage dependent T, N and L-type Ca^{2+} currents. They also contain a voltage-gated and TXX-sensitive Na^+ current that is activated during action potential and contributes to α-cells, in contrast to β-cells, overshooting action potentials (exceeding 0 mV) (Bokvist et al., 1999; Gopel et al., 2000; Yoshimoto et al., 1999). α-Cells also contain K_{ATP} channels of the same type as those that constitute the resting conductance of the β-cell (Bokvist et al., 1999).

Yet, with all this knowledge the metabolic regulation of glucagon secretion is not elucidated. However, several hypothesis have been put forward; these include paracrine inhibitory actions of Zn^{2+} or GABA secreted by β-cells (Rorsman et al., 1989), and paracrine inhibition of glucagon secretion by somatostatin released by neighbouring δ-cells (Cejvan et al., 2003).

Studies in isolated inside-out patches have revealed that the pancreatic α-cells are equipped with K_{ATP} channels (Bokvist et al., 1999). In pancreatic β-cells, glucose inhibits the K_{ATP} channels leading to stimulation of electrical activity and insulin secretion (Ashcroft and Rorsman, 1989). Yet, if closure of K_{ATP} channels in β-cells results in stimulation of insulin secretion how can closure of the same channels in α-cells inhibit glucagon secretion? It has been proposed that this is due to the

different electrophysiological properties of the α-cells and β-cells. Thus, whereas β-cell electrical activity and secretion is principally dependent on L-type Ca^{2+} channels, α-cell action potential firing involves voltage gated Na^{+} channels. Unlike the L-type Ca^{2+} channels, the Na^{+} channels undergo voltage-dependent inactivation; that is, they enter a non-conducting state when the voltage becomes too positive (Gopel et al., 2000). Closure of the K_{ATP} channels in response to high glucose with resultant membrane depolarization will therefore, contrary to observations in β-cells, result in reduction of action potential firing, and thus inhibition of glucagon secretion.

A low K_{ATP} channel density combined with a relatively high ATP:ADP ratio inside the α-cells already at low glucose concentrations (Detimary et al., 1998) is indeed a requirement for keeping the membrane potential sufficiently depolarized to allow regenerative electrical activity while preventing voltage-dependent inactivation of the ionic conductances involved in action-potential firing. The concept that membrane depolarization due to closure of K_{ATP} channels is involved in glucose-induced inhibition of glucagon secretion is supported by the findings that addition of a low concentration of extracellular K^{+} leads to a moderate membrane deporlazation but a marked reduction in spike amplitude and frequency and decreases glucagon secretion to the same extent as a maximally inhibitory concentrations of glucose (Gromada et al., 2004).

1.4 BIOLOGICAL ACTIONS OF INSULIN AND GLUCAGON AND RECEPTOR SIGNALLING

1.4.1 Insulin action on skeletal muscle, liver and adipose tissue

Insulin is the primary anabolic hormone promoting the storage of energy in the fed state. In response to nutrients, insulin secreted from the β-cell directly enters the portal circulation regulating liver carbohydrate and lipid homeostasis, by stimulating glycogen synthesis, lipogenesis, and lipoprotein synthesis, and by suppressing gluconeogenesis, glycogeolysis, and the secretion of very low density lipoproteins (VLDL) (Khan and Pessin, 2002). Many of these effects are mediated at the level of gene expression, with insulin having positive effects on genes encoding glycolytic and lipogenic enzymes, and negative effects on genes encoding gluconeogenic enzymes (O'Brien and Granner, 1996). The major cellular mechanism for disposal of exogenous glucose is insulin-stimulated glucose transport into skeletal muscle through the GLUT4 (Huang and Czech, 2007). Glucose transporter 4 is highly expressed in skeletal muscle, and is unique due to its mostly intracellular localization in the unstimulated state, being acutely redistributed to the cell membrane in response to insulin and muscle contractions (Bryant et al., 2002). In muscle and liver, glucose is converted to glycogen for storage, a process regulated by insulin. Insulin stimulates glycogen synthesis by activating protein phosphatase 1, which activates glycogen synthase, and inactivates glycogen phosphatase kinase 3, an inhibitor of glycogen synthase. In adipose tissue, insulin also promotes glucose transport via GLUT4 to promote the storage of intracellular triglycerides (Khan and Pessin,

2002).

1.4.2 The insulin receptor and signalling

The insulin receptor is present in nearly all vertebrate tissues at levels ranging from 40 receptors per cell in circulating erythrocytes to over 200 000 receptors per cell in adipocytes (Kahn and Folli, 1993; White and Kahn, 1994). Systemic ablation of the insulin receptor is lethal, however, tissue specific genetic alterations of the insulin receptor in the mouse has revealed sophisticated crosstalk between insulin target tissues, and distinct roles for this receptor in liver, adipose tissue, skeletal muscle, brain, and pancreatic β-cells (Kahn et al., 2000).

The insulin receptor is composed of two 723 amino acid α-subunits that are each linked to a 620 amino acid β-subunit and to each other by disulfide bonds. Both subunits are derived from a single proreceptor by proteolytic processing. The α-subunits are located entirely outside the cell and contain the insulin binding sites, whereas the intracellular portion of the β-subunit contains the insulin-regulated tyrosine protein kinase. The insulin receptor family contains two other structurally related molecules, the insulin-like growth factor (IGF-1) receptor and the insulin receptor related receptor, an orphan receptor for which no ligand and biological function has yet been identified (Schumacher et al., 1993).

The binding of insulin to the α-subunit of the insulin receptor induces conformational changes to the receptor, stimulating the intrinsic tyrosine kinase activity of the β-subunit. Insulin receptors transphosphorylate several immediate substrates including Insulin Receptor Substrate (IRS)1-4, Shc, and Gab1, Cbl, adaptor protein with pleckstrin homology and src homology 2 domains (APS), and $P60^{dok}$. Each of these provide specific docking sites for other signalling proteins containing Src homology 2 (SH2) domains leading to the activation of downstream signalling molecules.

The two main insulin signalling pathways identified and characterized are the Phosphoinositide-3 kinase (PI3K)/PI3K dependent kinase 1 (PDK1)/Protein kinase B (PKB) and p21ras/Mitogen Activated Protein Kinase (MAPK) pathway. PI3 kinase plays a pivotal role in the metabolic and mitogenic actions of insulin regulating components of GLUT4, protein kinase C (PKC), and glycogen synthase kinase 3 (GSK3), all of which are critical in insulin-mediated metabolic effects.

The identification of insulin activation of the p21ras/MAPK pathway is relative recent (Kozma et al., 1993). p21ras is tethered to the cell membrane, and this localization is essential for its biological function (Willumsen et al., 1984). When bound to guanosine triphosphate (GTP), p21ras is activated and triggers a large number of cellular responses to insulin through the MAPK (Kozma et al., 1993; Manchester et al., 1994; Hausdorff et al., 1994). Many targets of MAPK are in the nucleus, these include c-jun, c-myc, elk-1, and Nuclear Factor IL-6 (NF-IL6) transcription factors that are all phosphorylated and activated by MAPK (Pulverer et al., 1991; Derijard et al., 1994; Seth et al., 1991; Gille et al., 1992; Janknecht et al., 1993; Nakajima et al., 1993). The current understanding is that

insulin activation of the p21ras/MAPK pathway is related to the regulation of transcription factors and nuclear events that mediate its effects on cell proliferation.

1.4.3 Glucagon actions on the liver

The key biological actions of glucagon converge on regulation of glucose homeostasis through its actions on the liver. The primary effects of glucagon on these metabolic pathways is an increase in hepatic glucose output resulting from elevated glycogen breakdown (glycogenolysis), an increase in the synthesis of glucose from gluconeogenic percursors (gluconeogenesis), and a decrease in the rate of glucose catabolism (glycolysis). Due to the involvement of three different pathways, the changes induced by glucagon are exquisitely coordinated. Short-term changes acutely alter the catalytic activity of the key enzymes, and are mediated by protein phosphorylation or by allosteric regulation. Long-term changes result from the regulation of gene expression for enzymes in the glycolytic and gluconeogenic pathways. Glucagon actions on the liver are mediated by cAMP, and the effect of glucagon on glycogenolysis is geared to provide a rapid increase in hepatic glucose production that counteracts dropping plasma glucose levels. The action of glucagon begins with an elevation of cAMP which activates protein kinase A (PKA). The first step in glycogenolysis is the phosphorylation and activation of phosphorylase *b* kinase, which catalyses the phosphorylation of inactive phosphorylase *b* to generate its active form, phosphorylase *a*. This phosphorylation provides enough conformationl change to activate the enzyme (Sprang et al., 1988). Phosphorylase *a* cleaves glycogen to generate glucose-1-phosphate, which is available for glucose formation. Insulin provides an important inhibitory action on this system by reducing the level of cAMP through an increase in phosphodiesterase activity (Conti, 2000). To increase hepatic glucose production, glucagon inhibits glycolysis and increases gluconeogenesis. A key regulatory step acted on by glucagon that impacts both pathways is the modulation of the two separate catalytic activities mediated by the bi-functional enzyme 6-phosphofructose-2-kinase/fructose-2.6 bisphosphate. The bifunctional enzyme activity regulates the level of fructose-2,6 bisphosphate, which has major effects on both glycoysis and gluconeogenesis through its acute allosteric regulation of two enzymes, 6-phosphofructose-1 kinase (glycolysis) and fructose-1,6 bisphosphatase (gluconeogenesis). The overall effect is that high level of fructose-2,6 bisphosphate stimulates glycolysis and low level allows gluconeogenesis to proceed (Amatruda JM, 2003). Approximately 70% of basal hepatic glucose output is glucagon dependent (Cherrington et al., 1978), emphasizing the importance of a tight regulation of this hormone. In the postprandial state glucagon is suppressed (Tse et al., 1983), likely due to the effects of hyperglycemia and hyperinsulinimia (Baron et al., 1987), whereas the primary role of glucagon is preventing and correcting hypoglycemia (Rosen et al., 1984; Boyle et al., 1989).

1.4.4 The glucagon receptor and signalling

Based on structural and amino acid similarities, the glucagon recpetor has been classified as a G-protein-coupled receptor (GPCR) family B member, as part of a subgroup including receptors for GIP, GLP-1/2, growth hormone releasing hormone (GHRH), PACAP, vasoactive intestinal popypetide (VIP), and secretin (Gether, 2000). These receptors are characterized by their large extracellular domain, display little sequence identity to other GPCRs, and similar to their ligands are thought to share a common ancestral gene (Ulrich et al., 1998). The glucagon receptor shares the greatest amino acid sequence identity with the GIP receptor (GIPR) (44%) and the GLP-1 receptor (GLP-1R) (42%) (Usdin et al., 1993). The greatest variability between these receptors is in the intracellular carboxy-terminal tail region, while the extracellular N-terminus is relatively well conserved. Based on sequence analysis, the glucagon receptor encoded a 485 amino acid protein with a predicted molecular weight of approximately 55 kDa. Initial characterization of the glucagon receptor revealed widespread tissue distribution within the liver, kidney, spleen, thymus, adrenal glands, pancreas, cerebral cortex, lung, heart, adipose tissue, brain, and throughout the gastrointestinal tract (Dunphy et al., 1998; Svoboda et al., 1994). Further examination of the glucagon receptor expression in islet cells was carried out by Moens et al. (Moens et al., 1996) using FACS sorted rat α-cells and β-cells. Glucagon receptor mRNA was confirmed to be expressed in β-cells, with no significant levels in the non-β-cell sorted fraction (>80% α-cells, <10% β-cells). This finding was supported by studies on glucagonoma cells and functional receptor signalling data (Moens et al., 1996). As a Family B GPCR member, the glucagon receptor interacts with $G_{\alpha s}$ to stimulate an increase in cAMP through adenylate cyclase, and subsequent activation of PKA. This has been confirmed in rat liver cells (Birnbaum and Fain, 1977), pancreatic tumor cell lines (Goldfine et al., 1971), and islet β-cells (Moens et al., 1996). One set of downstream effectors are the MAP kinases, which include extracellular signal-regulated protein kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase/stress activated protein kinase. These protein kinases are critical points of convergence for cellular signal transduction pathways leading to cellular differentiation and proliferation (Robinson and Cobb, 1997).

1.5 REGULATION OF PANCREATIC ISLET CELL MASS

1.5.1 Pancreas development

The murine pancreas undergoes two waves of endocrine cell differentiation. The first wave gives rise to glucagon producing cells, followed by insulin producing cells often co-expressing glucagon (Teitelman et al., 1993). This led to the hypothesis that α-cells and β-cells develop from the same cell lineage, and further, that α-cells functions as precursor cells for β-cells, however, Herrera et al. have shown that these hormone co-expressing cells are a transient population, and do not contribute to mature islets (Herrera, 2000), furthermore, with respect to the cell lineage of endocrine cells,

Herrera et al. have shown that adult α-cells and β-cells derive from cells that have never transcribed insulin or glucagon respectively. The second wave of endocrine differentiation relies on the transcription factors Pdx-1 and hepatocyte nuclear factor 6 (Hnf6) (Jacquemin et al., 2000; Offield et al., 1996). Endocrine cells produced by the second wave form endocrine cords adjacent to ducts (Pictet et al., 1972), delaminate from the ductal epithelium, differentiate, proliferate, and then cluster to form islets expressing all five endocrine cell types. The basic helix-loop-helix (bHLH) protein Neurogenin3 (Ngn3) is required for the differentiation of pancreatic progenitor cells into endocrine cells, predominantly glucagon producing cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000). While Ngn3 promotes an endocrine commitment, the specification of the different cell types is controlled by other transcription factors (for reviews Collombat et al., 2006; Edlund, 2001). Focusing on α-cells, an increasing number of transcription factors have been identified as important for α-cell development through mouse knockout and transgenic approaches including Brain-4 (Brn-4) and POU homoedomain-containing protein, Pax6, and the aristaless-related homeobox (Arx). Brain-4 is a very early α-cell marker exclusively localized to the glucagon producing α-cells (Heller et al., 2004). It has been shown that Brn4 acts at an early developmental stage, however is not essential for α-cell fate (Hussain et al., 2002), and Brn4 knockout mice revealed that Brn4 is not a critical transcription factor for expression, biosynthesis, and secretion of glucagon *in vivo* (Heller et al., 2004). Pax6 knockout mice present with decreased number of α-cells or absence of α-cells (St-Onge et al., 1997), however, there exists data suggesting that α-cells are formed in normal numbers in Pax6 mutant mice, but these α-cells fail to transcribe the glucagon gene to any significant extent (Heller et al., 2004). Arx knockout mice exhibit increased numbers of β-cells and δ-cells at the expense of α-cells, suggesting that Arx is required for α-cell formation and repression of β-cells and δ-cells (Collombat et al., 2003). Arx actions are exactly opposite to those of Pax4, supported by increased Pax4 and Arx expression in Arx and Pax4 knockout mice respectively (Collombat et al., 2003), and by Pax4 knockout mice having increased numbers of α-cells at the expense of β-cells and δ-cells (Sosa-Pineda et al., 1997). In mature endocrine cells Arx expression is restricted to α-cells and δ-cells, whereas Pax4 is expressed in β-cells only (Grapin-Botton et al., 2001). Arx mediates activin A induced inhibition of α-cell proliferation, but not its ability to suppress glucagon gene expression (Mamin and Philippe, 2007). These effects of activin A in α-cells are opposite to those in β-cells where activin A (growth and differentiation factor) increases insulin and Pax4 gene expression (Demeterco et al., 2000; Zhang et al., 2001).

1.5.2 Regulation of pancreatic islet cell mass

Increasing evidence indicates that β-cells exist in a dynamic state (Bonner-Weir, 2001). Overall β-cell mass can be regulated by variations in cell number (hyperplasia), cell size (hypertrophy/atrophy), neogenesis/differentiation and apoptosis. β-Cell mass expands throughout adulthood (Butler et al., 2003b; Kushner et al., 2002), and in addition to this increase occurring

throughout life, there are dramatic compensatory changes in β-cell mass in response to changes in demand, such as during pregnancy (Rhodes, 2005), obesity (Butler et al., 2003a), and insulin resistance (Bruning et al., 1997). The ongoing debate in the field of β-cell mass regulation relates to the postnatal source of new β-cells, and whether new β-cells arise from self-duplication (replication, proliferation), or require specialized progenitor cells. Dor et al. have provided evidence, using a genetic lineage tracing approach in mice, that β-cells formed after birth, or following 70% partial pancreatectomy are generated by self-duplication (Dor et al., 2004; Teta et al., 2007). Opposing evidence favours a concept in which pancreatic ductal epithelial cells have the potential to regress to a less differentiated progenitor cell capable of producing new islets and acini (Bonner-Weir et al., 2004). However, genetic lineage tracing has not confirmed this hypothesis yet. Finally, there also exists evidence that other progenitor cells or stem cells contribute to β-cell proliferation (Xu et al., 2008).

In general, little is known about the potential of β-cell replication and/or neogenesis in human islets. Fully differentiated FACS sorted human adult insulin-producing β-cells do not proliferate *in vitro* (Parnaud et al., 2008), whereas β-cell proliferation in intact human islets *in vitro* has been reported (Maedler et al., 2006; Shu et al., 2008). The curative potential in understanding how β-cells are generated has led to a major interest in this field, with an exclusive focus on the β-cell. However, virtually nothing is known about the regulation of pancreatic α-cell mass leaving a critical knowledge gap in the area of islet cell mass regulation and growth.

1.5.3 Signals inducing β-cell neogenesis and proliferation

Numerous β-cell growth factors have been identified for both precursor and adult β-cells, including growth hormone (GH), prolactin (PRL), insulin, insulin-like growth factor (IGF)-1, the incretins GLP-1 and GIP, and exendin-4 (GLP-1 receptor agonist) (Friedrichsen et al., 2003; Lingohr et al., 2002; Nielsen et al., 2001).

Growth hormone and PRL act on tyrosine kinase receptors, signaling via Janus Kinase (JAK)2 /signal transducers and activators of transcription (STAT)5a/b to regulate β-cell mitogenesis. The known actions of GH and PRL are mediated via increased expression of factors that promote replication, including preadipocyte factor-1 (Pref-1) (Nielsen et al., 2001), cyclin D2 (Friedrichsen et al., 2003), and the anti-apoptotic protein Bcl-xL and members of the suppressors of cytokine signaling (SOCS) family (Grad et al., 2000; Nielsen et al., 2001). Survival actions of IGF-1 are via PI3K-dependent PKB signalling, which has been shown to act on numerous effectors important in cell survival including GSK-3, β-catenin, murine double minute (Mdm)2, procaspase-9, Retinoblastoma (Rb) protein, and forkhead family transcription factor (FKHR)-1 (reviewed in Lingohr et al., 2002).

The signalling pathways activated by GLP-1, GIP, and the GLP-1 receptor agonist exendin-4 in β-cells depend on the initial production of cAMP (Widmann et al., 1994). Protein kinase A-mediated phosphorylation of the transcription factor cAMP responsive element binding protein

(CREB) is the classical mode of transcriptional regulation by GLP-1 and GIP. This pathway is also involved in glucose mediated β-cell survival (Costes et al., 2006). GLP-1 and GIP also activate the ERK1/2 pathway (Ehses et al., 2002; Gomez et al., 2002), probably through a combination of increased cAMP and $[Ca^{2+}]i$ activating the protein kinase Raf (Arnette et al., 2003; Trumper et al., 2005). Moreover, GLP-1 and exendin-4 have been shown to activate the Wnt signaling through the GLP-1 receptor coupled to avtivation of PKA/cAMP, and the prosurvival kinase PKB (Liu and Habener, 2008).

1.5.4 Signals inducing and preventing β-cell death

Various inducers of β-cell apoptosis have been identified. With respect to nutrient overload, chronically elevated glucose levels and saturated free fatty acid (FA) palmitate levels are factors causing β-cell apoptosis (Donath et al., 1999)) (Maedler et al., 2003; Maedler et al., 2001; Prentki and Nolan, 2006). In fact, there is evidence that cytokines produced by β-cells in response to hyperglycemia and/or hyperlipidemia (Ehses et al., 2007; Maedler et al., 2002), may contribute to this β-cell apoptosis. More recent evidence indicates that endoplasmatic reticulum (ER) stress, activating the Unfolded Protein Response (UPR) may cause β-cell apoptosis (Scheuner et al., 2005). Thus, a nutritional overload, leading to enhanced metabolic demand, seems to be detrimental to β-cell survival.

Little is known about the signalling mechanisms that cause β-cell apoptosis due to nutrient overload, although downstream activation of stress MAP kinases, cJun kinase (Jnk)-1/2 and p38 may be involved (Maedler et al., 2008; Rhodes, 2005). For cytokine induced apoptosis, the activated pathway depends on the specific cytokine. IL-1β signals through MyD88, activating Interleukin-1 Receptor Activated Kinase (IRAK) and Tunor Necrosis Factor Receptor (TNFR)- associated factor (TRAF)-6, and resulting in activation of MAPK, JNK1/2, and p38 (Lee and Lee, 2002; Levine and Yuan, 2005) leading to activation of transcription factors that contribute to increased apoptotic gene expression(Bradley and Pober, 2001; Lee and Lee, 2002). Interleukin-1β induced apoptosis has furthermore been shown to involve NF-KappaB signalling (Lee and Lee, 2002; Mercurio and Manning, 1999).

In comparison to the mitogenic effects of GLP-1, GIP, and exendin-4, their anti-apoptotic effects are less well described. However, studies of the signalling pathways mediating the anti-apoptotic actions support a role for PI3K, PKB, cAMP, and MAPK, in part via decreased levels of caspase-3 and increased levels of Bcl-2, and Bcl-XL (Ehses et al., 2003; Farilla et al., 2002; Hui et al., 2003; Wang and Brubaker, 2002). Protection of nutritional overload induced apoptosis in human islets was shown to involve Akt/PKB and nuclear factor–kappaB activation (Buteau et al., 2004). More recent evidence implicates a role for the transcription factor cAMP response element binding protein as a downstream mediator of the anti-apoptotic actions of GLP-1, through a pathway involving cAMP

response element binding protein-mediated induction of Akt via the insulin signaling protein IRS-2 (Jhala et al., 2003). GIP stimulates the expression of the anti-apoptotic Bcl2 gene in β-cells through a pathway involving AMP-activated protein kinase (AMPK), cAMP-response CREB coactivator 2 (TORC2) and CREB (Kim et al., 2008b).

A thorough literature search for studies examining the regulation of α-cell fate did not reveal a single study, revealing a critical knowledge gap in the research field of diabetes, and furthermore emphasizes the importance of the present work.

1.6 PANCREATIC α-CELL REGULATION OF THE β-CELL

1.6.1 Do the α-cell and glucagon have a role in pancreas development and growth?

Little is known about the role of the pancreatic hormones in pre- and postnatal islet cell growth and development. Already in 1970 Rall et al. speculated that glucagon was necessary for the induction of differentiation of the other hormone cell lineages during an early phase of pancreatic differentiation. The speculation was mainly based on the finding that glucagon is the first hormone to be expressed in the embryonic pancreas. Indeed, since 1970 several pieces of evidence have provided a role for glucagon signalling in insulin positive cell differentiation. Pax6 knockout mice lack glucagon producing α-cells, and show a delay and a deficit in insulin positive cell differentiation (St-Onge et al., 1997). *In vitro* inhibition of proglucagon, in the early embryonic pancreas, leads to the absence of the early first wave insulin positive differentiation, an observation rescued by exogenous glucagon (Prasadan et al., 2002). In support of these findings, PC2 (necessary for the production of mature glucagon) knockout mice lack early insulin positive cells (Vincent et al., 2003). Further evidence for a role of glucagon in β-cell differentiation is provided with the glucagon receptor knockout mice, also in these mice there is a delay in the differentiation of insulin producing cells (Vuguin et al., 2006). In addition, glucagon producing cells from these mice express Pdx-1 and GLUT2 indicative of an immature cell. Also PC2 knockout mice have signs of immature cells expressing both glucagon and insulin. Thus, glucagon receptor signalling in the early pancreas appears to have a paracrine role in the induction of either pancreatic endocrine progenitor cells to initiate a differentiation program toward β-cells, as well as to further mature these cells. Glucagon also seems to have an autocrine function. In the absence of a glucagon signal to the glucagon producing cells, these cells appear to be frozen in an immature state (Gittes and Fisher, 2006).

The glucagon receptor knockout mice have provided additional insight into the role of glucagon signalling in the regulation of islet cell composition, proliferation and apoptosis. The absence of glucagon signalling leads to an increased percentage of α-cells and δ-cells per islet (Gelling et al., 2003; Vuguin et al., 2006). Islet architecture is partly disrupted, with islets displaying a thick mantle comprised of α-cells and δ-cells, and an abnormal distribution of δ-cells scattered in the

core of the islet. β-Cell mass is increased as a consequence of increased islet number. The increased α-cells and β-cell mass is due to hypertrophy and hyperplasia respectively (Gelling et al., 2003; Vuguin et al., 2006) in contrast to proliferation, the rate of islet cell apoptosis was found to be very low.

An observation substantially adding to the relevance of glucagon signalling is based on an individual carrying a homozygous point mutation in the glucagon receptor gene resulting in ablation of receptor activity. This individual displayed an almost identical phenotype with increased α-cell hyperplasia (Gelling et al., 2003).

Thus, glucagon signalling plays an important role in the development of mature α-cells and β-cells, and in the regulation of islet cell composition and mass.

1.6.2 Do the α-cell and glucagon have a role in β-cell secretory function

Already in 1966 glucagon was described to have insulinotropic effects in humans (Samols et al., 1966). Today, it is known that the pancreatic α-cell helps to maintain β-cell glucose-competence via glucagon (Huypens et al., 2000) acting through both glucagon receptors and GLP-1 receptors on β-cells (Moens et al., 1998). The concept that glucose responsiveness of β-cells depends on sufficient intracellular production of cAMP has been supported by various *in vitro* models (Gorus et al., 1984; Holz et al., 1993; Pipeleers et al., 1985c). Rat islets from the dorsal, glucagon rich, pancreas had improved insulin response to glucose in comparison to islets from the ventral lobe (Pipeleers et al., 1985c) suggestive of a paracrine role of glucagon on β-cell glucose responsiveness. FACS sorted purified rat β-cells, separated from neighboring glucagon-producing α-cells, display a poor secretory response to glucose (Pipeleers et al., 1985c), despite normal rates of glucose metabolism (MacDonald, 1990; Schuit et al., 1988) and glucose induced insulin bio-synthesis. Exogenous glucagon restored β-cell insulin secretion (Pipeleers et al., 1985b) and increased cAMP concentrations to values observed in islets (Schuit and Pipeleers, 1985). Thus, these observations suggest that normal insulin secretion from β-cells is not only dependent on glucose recognition, but also on a certain threshold of intra-islet glucagon and activation of glucagon receptors on β-cells (Huypens et al., 2000). Furthermore, glucagon receptor knockout mice have impaired β-cell function, and an impaired insulin response to the non-glucose stimuli carbachol, arginine, CCK-8, GLP-1, and GIP (Sorensen et al., 2006). The fact that these secretagogues signal to insulin secretion through different pathways suggests that disruption of glucagon signalling influences late events in the insulin secretory pathway (Sorensen et al., 2006). The insulin response to forskolin was markedly reduced in glucagon receptor knockout islets, indicating that cAMP may be downregulated as a consequence of the lack of glucagon signalling. The recent establishment of a β-cell overexpressing glucagon receptor transgenic mouse confirms this paradigm, since these mice have improved glucose tolerance and increased insulin secretion in response to glucose (Winzell et al., 2007).

1.6.3 Does the α-cell regulate β-cell mass?

In addition to glucagon effects on the β-cell, the possibility that the α-cell ever produces GLP-1 in the context of islet development or regeneration has been addressed by several studies demonstrating induction of PC1/3 responsible for liberating GLP-1 from proglucagon expression in α-cells. Pancreatic α-cell expression of PC1/3 is induced in rats given streptozotocin (STZ), leading to significant increases in the levels of bioactive GLP-1 in the rat pancreas (Nie et al., 2000). Proglucagon and PC1/3 are expressed in the embryonic mouse pancreas from E10.5 to E15.5 (Wilson et al., 2002) raising the possibility that bioactive GLP-1 might be liberated from these cells with potential implications for β-cell growth and development. Similarly, neonatal rats treated with STZ exhibit β-cell regeneration and increased pancreatic levels of GLP-1. Further, the antagonist exendin(9-39) diminished β-cell regeneration observed after STZ administration (Thyssen et al., 2006). These studies however, do not prove that bioactive GLP-1 exerts a role during development or regeneration, but they certainly expand the concept to include a potential for some islet α-cells to produce GLP-1 in a developmental or experimental setting in the context of β-cell injury. GLP-1 production from islet α-cells has also been observed following complete or partial reduction of glucagon receptor signalling. Mice with targeted disruption of the glucagon receptor gene, or rodents with reduced glucagon receptor expression develop α-cell hyperplasia and increased pancreatic and plasma GLP-1 (Gelling et al., 2003; Sloop et al., 2004). Supporting the concept that α-cell-derived GLP-1 may have a role in β-cell survival, a recent study showed enhanced islet survival after transplantation when α-cells overexpressed PC1/3 leading to increased production of GLP-1 and GLP-2 (Wideman et al., 2007).

1.7 DIABETES MELLITUS

Diabetes is a metabolic disease affecting over 150 million people worldwide, a number expected to double by 2025 (Zimmet et al., 2001). The disease is characterized by disordered metabolism and abnormally high blood sugar. Current classifications distinguish between type 1 diabetes, which is characterized by autoimmune β-cell destruction, and the broader type 2 diabetes, which is defined as ranging from predominantly insulin resistance with relative insulin deficiency, to predominantly an insulin secretory defect with insulin resistance (2006). However, increasing clinical evidence is highlighting overlap between these two diabetic conditions. For example, immunological phenomena classically associated with T1DM such as anti-islet cell antibodies, and elevated systemic levels of cytokines and chemokines are also present in T2DM (Kolb and Mandrup-Poulsen, 2005; Pietropaolo et al., 2000). Moreover, obesity, which is associated with insulin resistance and T2DM, shows strong correlations with the increasing incidence of T1DM (Hypponen et al., 2000; Kibirige et al., 2003). The emphasis in this thesis is on T2DM, yet a very brief introduction on T1DM follows.

1.7.1 Type 1 diabetes

Type 1 diabetes comprises 5 - 10% of all cases of diabetes (2006). The disease is characterized by complete or near complete insulin deficiency caused by an immune mediated selective destruction of the insulin producing β-cells (from (Kristiansen and Mandrup-Poulsen, 2005), and by absolute or relative hyperglucagonemia (Muller et al., 1970; Unger, 1971). Type 1 diabetes can be considered an inflammatory disease of the pancreatic islets, in which a process of programmed cell death (apoptosis) is elicited in the β-cells by interaction of activated T-cells and proinflammatory cytokines in the immune infiltrate (Eizirik and Mandrup-Poulsen, 2001). The immune mediated β-cell destruction, marked by the production of autoantibodies to the β-cell (islet cell autoantibodies ICA, insulin autoantibodies IAA, glutamic acid decarboxylase GAA or GAD, and protein tyrosine phosphatase IA2 or IAC512), is thought to be initiated by interaction between yet unknown environmental factors and type 1 diabetes susceptibility gene variants (Atkinson and Eisenbarth, 2001).

Pancreatic islet morphology in T1DM is characterized by small islets, a reduced islet volume/mass (Alberti and Zimmet, 1998; Atkinson and Eisenbarth, 2001), decreased or no β-cell mass (Alberti and Zimmet, 1998), and an increase in the relative proportion of glucagon producing α-cells (Gepts and De Mey, 1978; Gepts and Lecompte, 1981). Insulitis of islets, defined as islets infiltrated with T lymphocytes (CD8+ and CD4+) (Foulis et al., 1986; Imagawa et al., 1999), B lymphocytes and macrophages (Foulis, 1989) is a common observation in recent onset of T1DM, and occurs only in islets with β-cells.

1.7.2 Type 2 diabetes

Type 2 diabetes accounts for 90 – 95% of all the cases of diabetes, and is highly associated with obesity. Type 2 diabetes (and T1DM) is evident then fasting plasma glucose levels are ≥ 7.0 mM. The disease is considered a heterogeneous disease characterized by failure of the β-cells to compensate for peripheral insulin resistance. The disease can range from predominantly insulin resistance with relative insulin deficiency, to predominantly an insulin secretory defect with insulin resistance (2006). In addition to the well-recognized impairment in β-cell secretory function (Polonsky et al., 1988), a decrease in β-cell mass, due to increased β-cell apoptosis, is thought to contribute to the pathogenesis of type 2 diabetes, as has been shown in humans and rodent models of the disease (Butler et al., 2003a; Donath and Ehses, 2006; Masiello, 2006; Portha, 2005). However, it remains unclear whether patients with T2DM have a lower β-cell mass early in life, fail to increase their β-cell mass in the face of insulin resistance, and/or have progressive β-cell loss throughout life (Rhodes, 2005)

Investigations on the pathophysiology of T2DM have mainly focussed on the β-cell, mostly neglecting the α-cell. However, in the early 1970's numerous important findings on glucagon

physiology took place, and as a consequence, the interest in a role for the α-cell and glucagon in diabetes increased dramatically. Among the most important discoveries was the suppression of glucagon in response to increased glucose (Muller et al., 1970; Unger, 1971). This physiologcical response is impaired or lacking (Basu et al., 2004; Shah et al., 2000) in patients with T2DM. Accordingly, patients with T2DM present with absolute or relative hyperglucagonemia (Muller et al., 1970; Unger, 1971), defined as inappropriately elevated glucagon levels for the prevailing glycemia (Ward et al., 1984). In line with a dysfunctional α-cell, glucagon levels increase in response to amino acids in type 2 diabetic patients (Ward et al., 1984), and insulin withdrawal in a hyperglycaemic state is associated with hyperglucagonemia (Dobbs et al., 1975; Unger, 1971). Thus, there exists ample evidence that the pancreatic α-cell and glucagon secretion are dysregulated in T2DM, however glucagon infusion into healthy individuals and people with diabetes revealed that hyperglucagonemia does not cause glucose intolerance in healthy people or bring about deterioration of diabetic control when insulin is available. Hyperglucagonemia only in the insulin deprived state can worsen the diabetic condition (Sherwin et al., 1976). These findings suggest a primary role of insulin deficiency in the diabetogenic action of glucagon.

Together with the dysregulation in glucagon secretion there is an expansion of the relative proportion of α-cell volume/area/mass in patients with T2DM (Deng et al., 2004; Orci et al., 1976; Yoon et al., 2003), and in glucose intolerant C57BL6 mice fed a HF diet (Sauter et al., 2008). However, a decrease in α-cell volume has also been reported in patients with T1DM diabetes (Sayama et al., 2005). Thus, at the level of the pancreatic islet T2DM is characterized by a dysregulated secretion of both insulin and glucagon, as well as by pronounced alterations in islet cell morphology and mass.

1.7.3 Insulin resistance in type 2 diabetes

All patients with T2DM present with insulin resistance, which manifests at the level of the skeletal muscle, adipose tissue, and liver, however, only 10% of people who are insulin resistant are diabetic (de Luca and Olefsky, 2008) indicating an enormous capacity of the β-cell to adapt to the increased insulin demand. Peripheral insulin resistance refers to a target tissue defect in insulin action, manifesting as an impaired ability of insulin to stimulate overall glucose disposal in skeletal muscle and adipose tissue, and inhibit glucose output from the liver, resulting in hyperglycemia.

Insulin stimulated glucose transport in skeletal muscle and adipocytes is decreased in obesity and in patients with T2DM (Kim et al., 1999a). The impaired glucose transport is due to decreased insulin induced GLUT4 translocation in skeletal muscle. The regulatory unit p85 of PI3K associates with IRS-1 and IRS-2 in response to insulin, and is essential for GLUT4 translocation and glycogen synthase activity, both of which are impaired in skeletal muscle of patients with T2DM (Cusi et al., 2000; Kim et al., 1999a). Moreover, patients with T2DM reveal an impaired ability of insulin to

stimulate IRS-1 and IRS-2 phosphorylation in adipocytes (Thies et al., 1990) and skeletal muscle (Bjornholm et al., 1997).

Hepatic insulin resistance leads to impaired inhibition of hepatic glucose output, which combined with increased glucagon levels and increased gluconeogenesis leads to increased basal rates of hepatic glucose output, a feature of type 2 diabetes (Ferrannini and Groop, 1989).

1.7.4 Systemic and pancreatic inflammation in type 2 diabetes

Obesity is strongly associated with the development of insulin resistance (Mokdad et al., 2003), and is the main risk factor for the development of T2DM. A chronic, low-grade inflammatory state is present in obesity, with adipose tissue macrophage infiltration and proinflammatory activity of macrophages (Neels and Olefsky, 2006). Adipocytes and macrophages have been proposed to be the origin of elevated circulating levels of various cytokines and chemokines, such as IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and tumor necrosis factor (TNF)α observed in patients with T2DM (Neels and Olefsky, 2006). Epidemiological studies suggest that low-grade inflammation precedes and predicts the development of T2DM (Festa et al., 2002). Proinflammatory cytokines can cause insulin resistance (Hotamisligil et al., 1993), and impair β-cell function (Maedler et al., 2002), and anti-inflammatory mediators may reverse both (Larsen et al., 2007; Yuan et al., 2001), implying that inflammation may be directly involved in the pathogenesis of T2DM.

Inflammation is defined as the local response to tissue injury. It is characterized by immune cell invasion and local release of cytokines and chemokines and is sometimes accompanied by functional or structural damage of the invaded tissues. It is not in itself a disease, but a manifestation of disease. Inflammation has beneficial effects such as preventing spread of infections or promoting regeneration. Equally, it may exacerbate disease by tissue destruction due to inflammatory mediators, reactive oxygen species, and complement components (Donath et al., 2008).

The histology of islets from patients with T2DM display an inflammatory process evidenced by the presence of cytokines, apoptotic cells, immune cell infiltration, amyloid deposits, and eventually fibrosis. Pancreatic β-cells are able to produce and secrete IL-1β, and a nutritional metabolic overload in the form of excess glucose increases the production of IL-1β. Furthermore, IL-1β expression is increased in β-cells in patients with T2DM, and treatment of these patients with the naturally occurring IL-1 antagonist IL-1Ra leads to a decrease in glycated haemoglobin A1c (HbA1c), improved β-cell function, and significantly reduced IL-6 levels (Larsen et al., 2007). In high fat (HF) fed mice, IL-1Ra treatment improved glycemia, and glucose tolerance (Sauter et al., 2008). Similarly, elevated glucose has been shown to increase secretion of a novel β-cell cytokine PANDER (FAM3B), and *in vitro* studies have confirmed that human and mouse islets, as well as isolated rodent β-cells and β-cell lines (MIN-6 and INS-1) produce various cytokines and chemokines (Ehses et al., 2007; Frigerio et al., 2002; Yang et al., 2005). Among the chemokines secreted in response to a nutritional

overload is IL-8, which caused an increased migration of monocytes and neutrophils. The possibility exists that locally produced IL-8 may promote a concentration gradient leading to monocyte/granulocyte transmigration and infiltration into islets.

Islet derived inflammatory factors and islet inflammation may exert beneficial and/or deleterious effects on islet function and survival. Low concentrations of IL-1β promote β-cell function and survival, whereas high concentrations lead to impaired β-cell function and survival, indicative of a very sensitive system where doses and time of exposure are critical for whether the outcome is deleterious or reparative.

The presence of inflammatory cells in islets in T2DM was until recently never investigated, and pancreatic islet inflammation in T2DM is thus a recent concept in the context of T2DM. Immune cell infiltration is indeed present in various rodent models of T2DM and in patients with T2DM (Ehses et al., 2007; Homo-Delarche et al., 2006). The number of islet-associated Cd11b+ cells is increased in high fat diet fed C57BL/6 mice already after 8 weeks on HF diet, and in the spontaneous model of T2DM the db/db mouse. Human diabetic islets present with increased numbers of CD68+ cells/islet compared to non-diabetic patients. In some cases type 2 diabetic islets showed intra islet invasion of CD68+ cells (in contrast to perivascular location), which was associated with decreased insulin staining and significant amyloid deposits. Type 2 diabetic islets characterized by increased CD68+ cells did not show increased TUNEL+ cells, and macrophages were never observed in the vicinity of TUNEL+ cells. The macrophages were positive for HLA-2 and CD163 indicating a status of activation. This suggests that an inflammatory process is an early event in T2DM pathophysiology and probably contributes to the progression of the disease. Thus, local islet inflammation might be a general phenomenon in T2DM, in addition to systemic and adipose tissue inflammation.

1.8 INTERLEUKIN-6 AND ITS RECEPTOR

1.8.1 Interleukin-6

While a number of interleukins such as IL-1 and IL-10 are pleiotrophic in their effects (Dinarello, 1996), IL-6 may be considered the prototypical pleiotrophic cytokine (Kishimoto, 1989). This is reflected in the variety of names originally assigned to IL-6 based on function; interferon-β2, IL-1 inducible 26 kD protein, hepatocyte stimulating factor, cytotoxic T cell differentiation factor, B cell differentiation factor (BCDF) and/or B cell stimulatory factor 2 (BSF2) (Kishimoto, 1989). Once all the actions associated with the various names for IL-6 became connected with one common gene, the name IL-6 was proposed for this molecule (Poupart et al., 1987).

Interleukin-6 belongs to the IL-6 family of cytokines, including IL-11, oncostatin M, leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1, and cardiotrophin-like cytokine (Kamimura et al., 2003). The human IL-6 gene is approximately 5 kb long and consists of five exons (Yasukawa et al., 1987), and is located on chromosome 7p21 (Bowcock et

al., 1988). The cDNA of then BSF-2 (IL-6) was cloned in 1986. The amino acid sequence of human IL-6 consists of 212 amino acids with a signal peptide of 27 amino acids and two potential N-linked glycosylation sites (Hirano et al., 1986). IL-6 homologs have been reported in more than a dozen vertebrate species, and the mouse IL-6 protein is 42% identical to the human form and contains several potential O-linked glycosylation sites instead of the N-linked glycosylation site (Van Snick et al., 1988). The human IL-6 protein has a molecular weight ranging from 21 to 28 kDa, and can undergo posttranscriptional modifications such as glycosylation and serine phosphorylation (May et al., 1988), however, glycosylation does not seem to be necessary for its biological activity (Tonouchi et al., 1988). The x-ray crystal structure of human IL-6 shows a four-helix bundle, consisting of two pairs of antiparallel α-helices with up-up-down-down orientation, whose folding is conserved among cytokine family members (Kamimura et al., 2003). Based on the length of the helices, IL-6 is grouped into the "long chain" cytokines, which include growth hormone, erythropoietin, and granulocyte colony-stimulating factor.

Numerous cell types are reported to produce IL-6, these include T cells, B cells, polymorphonuclear cells, eosinophils, monocytes/macrophages, mast cells, dendritic cells, chondrocytes, osteoblasts, endothelial cells, skeletal and smooth muscle cells, islet β-cells, thyroid cells, fibroblasts, mesangial cells, keratinocytes, microglial cells, astrocytes, and certain tumor cells (Kamimura et al., 2003). Recently adipose tissue and skeletal muscle have been identified to produce IL-6 (Pedersen et al., 2001), leading to the proposition of IL-6 as an adipokine and a myokine (Pedersen and Fischer, 2007). Adipose tissue contributes as much as 35% to basal circulating IL-6 levels (Mohamed-Ali et al., 1997), whereas the production of IL-6 by skeletal muscle is induced in response to exercise (muscle contraction) 100-fold and up to a 100-fold increases in interstitial and circulating IL-6 have been reported respectively (Rosendal et al., 2005; Steensberg et al., 2002).

1.8.2 The interleukin-6 receptor and the signal transducer gp130

The receptor complex mediating the biological actions of IL-6 consists of two distinct membrane-bound glycoproteins, the 80 kDa cognate receptor subunit IL-6 receptor α (hereafter IL-6 receptor; also known as gp80, or CD126) and the 130 kDa signal transducing element gp130 (IL-6Rβ, CD130)(Jones et al., 2001). The IL-6 receptor and the gp130 belong to the type I cytokine receptor family, which in addition to the IL-6 family of cytokines, comprises leptin, GH, PRL, erythropoietin (EPO), thrombopoietin (THPO), and granulocyte- and granulocyte/macrophage-colony stimulating factors (GM-CSF) (Kamimura et al., 2003).

The human IL-6 receptor gene maps to chromosome 1q21 (Online Mendelina Inheritance in Man x147880), and the cDNA of the IL-6 receptor was cloned in 1988 (Yamasaki et al., 1988). The amino acid sequence of the human IL-6 receptor is 468 amino acids including a signal peptide of 19 amino acids. As with other cytokine type I receptor family members, the extracellular region has four conserved cysteine residues plus a WSxWS motif. In addition, the receptor has an Ig-like domain at its

N-terminus (Yamasaki et al., 1988). The short cytoplasmic domain does not include a tyrosine kinase domain, and is not required to initiate intracellular signalling (Yamasaki et al., 1988). In order for IL-6 to induce signaling, IL-6 binds to the IL-6 receptor with a Kd = 5.5 nM, and this low affinity complex subsequently recruits a gp130 molecule to form a high affinity complex with a Kd = 50 pM (Hibi et al., 1990). Mouse and rat IL-6 receptor are 54% identical to human IL-6 receptor in amino acid sequence, with 50% identity in the extracellular region (Sugita et al., 1990).

Due to the existence of a soluble IL-6 receptor the number of cell types expressing the IL-6 receptor does not reflect the spectrum of cell types that can respond to IL-6. Cells known to express the IL-6 receptor include CD4+ and CD8+ T cells, hepatocytes (Geisterfer et al., 1993), CD34+ stem cells (Tajima et al., 1996) neurons (Schobitz et al., 1993), neutrophils (Modur et al., 1997), monocytes (Horiuchi et al., 1994), and osteoblasts (Udagawa et al., 1995). The IL-6 receptor is furthermore found expressed in intestinal epithelial cells (Shirota et al., 1990), in the pituitary and adrenal cortex (Bethin et al., 2000), in leukocyten (Udagawa et al., 1995), adipose tissue (Memoli et al., 2007), and in skeletal muscle (Keller et al., 2005).

Soluble 55 kDa forms of the IL-6 receptor (sIL-6 receptor) are generated by proteolytic cleavage (Mullberg et al., 1994) of the membrane bound form, or by alternative splicing of its mRNA (Horiuchi et al., 1994). The soluble IL-6 receptor acts in an agonistic manner when bound to IL-6, subsequently interacting with cells expressing only gp130. Thus, in association with sIL-6 receptor, IL-6 can signal in most tissues of the body. In healthy individuals the concentration of sIL-6 receptor is approximately 75 ng/ml (Honda et al., 1992), and the sIL-6 receptor binds IL-6 with comparable affinity as the membrane associated IL-6 receptor (Memoli et al., 2000).

The use of the gp130 signal transducer is common for all cytokines belonging to the IL-6 family of cytokines; IL-11, oncostatin M, LIF, CNTF, cardiotrophin-1, and cardiotrophin-like cytokine. The human gp130 gene maps to chromosome 5q11(Rodriguez et al., 1995), and the cDNA of the gp130 was cloned in 1990 (Hibi et al., 1990). The amino acid sequence of the human mature gp130 consists of an extracellular region of 597 amino acids, a membrane spanning region of 22 amino acids, and a cytoplasmic region of 277 amino acids. Mouse gp130 has been cloned and found to be 77% identical to human gp130 at the amino acid level, 72% identity exists in the extracellular region.

In contrast to the IL-6 receptor, the cytoplasmic domain of gp130 contains several potential motifs for intracellular signaling, such as the YSTV sequence for Src homology 2-containing tyrosine phosphatase 2 (SHP-2) recruitment, and YXXQ motifs (where X means any amino acids) for STAT activation. Gp130 does not have an intrinsic kinase domain (Hibi et al., 1990). Instead like other cytokine receptors, the cytoplasmic domain of gp130 contains regions required for its association with the nonreceptor tyrosine kinase JAK, by which downstream signaling cascades are initiated.

Narazaki et al. (1993) purified 2 soluble forms of gp130 (sgp130) with a molecular mass of 90 and 110 kDa (Narazaki et al., 1993). Soluble gp130 is generated from alternative splicing of its RNA, and in contrast to sIL-6 receptor, sgp130 acts in an antagonistic manner (Tanaka et al., 2000). Soluble

gp130 does not bind sIL-6 receptor unless IL-6 is present. Soluble gp130 exists at concentrations approaching 400 ng/ml in plasma from healthy individuals. The expression of membrane bound gp130 is ubiquitous (Saito et al., 1992).

A scenario for the IL-6/IL-6 receptor/gp130 interaction cascade can be seen in Figure 1 and depicted as follows: 1) IL-6 binds to the IL-6 receptor forming a heterodimer, 2) the binary IL-6/IL-6 receptor complex contacts the gp130 cytokine binding modules and also forms contacts between the C-terminal cell surface domain of IL-6 receptor and gp130, resulting in a trimolecular complex with a 1:1:1 stoichiometry, which is not yet able to generate a signal 3) two trimolecular complexes are assembled together completing a hexameric complex, which is competent to generate intracellular signals (Chow et al., 2001).

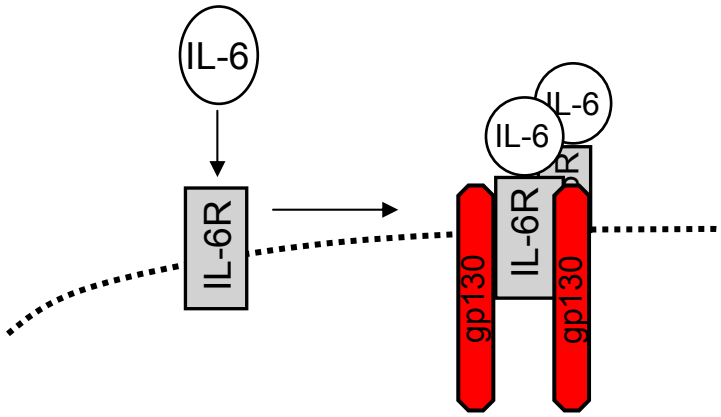


Figure 1: Two trimolecular complexes each with a 1:1:1 stoichiometry of the IL-6/IL-6R/gp130 completing a hexameric complex competent to generate intracellular signals.

1.8.3 Interleukin-6 receptor signal transduction

Interleukin-6 induced, gp130 mediated JAK activation initiates two distinct signaling pathways: 1) the JAK/STAT pathway, and 2) SHP-2/ERK/MAPK pathways (Kristiansen and Mandrup-Poulsen, 2005). In brief, the IL-6/IL-6 receptor/gp130 hexameric signalling complex brings the JAK kinases into close proximity, resulting in their transactivation of each other. The activated JAK kinases phosphorylate tyrosine residues in the cytoplasmic domain of gp130. The human gp130 cytoplasmic domain has 6 tyrosine residues, where the second tyrosine residue Y759 is required for gp130 mediated phosphorylation of *Src* homology 2-containing tyrosine (SHP-2). The third to sixth tyrosine residues (Y767, Y814, Y905, and Y915 in human gp130) form YXXQ motifs; these motifs are responsible for the activation of the transcription factor STAT (Fukada et al., 1996) (Gerhartz et al., 1996; Yamanaka et al., 1996). Hirano et al. (Hirano et al., 1997) have provided evidence that the distinct intracellular signaling pathways activated by gp130 may result in a variety of physiological events such as cell proliferation, differentiation, survival and apoptosis (Jones et al., 2001; Kamimura et al., 2003). The final biological output depends on the signalling pathway that prevails in the target

cell, which further may be controlled by the metabolic state of the cell, and a variety of other stimuli, a concept Hirano et al. proposed to call “the signal orchestration model” (Kamimura et al., 2003).

The simultaneous generation of contradictory signals is not unique to gp130 singal transduction. The binding of TNFα to TNFR1 induces a cascade of caspases resulting in apoptosis of the target cell; however, signal transduction via TNFR1 also activates transcription factors (AP-1 and NF-kB) resulting in antiapoptotic responses (reviewed in Baud and Karin, 2001). Furthermore, cyclin D1 and the cyclin dependent kinase inhibitor p21 (cyclin D1 is negatively regulated by p21) can both be activated by growth factors. These observations support the notion that certain cytokines and growth factors have the potential to simultaneously activate contradictory signals within a target cell. A mechanism possibly explaining how a pleiotropic cytokine like IL-6 can exert multiple functions in a variety of cells (Kamimura et al., 2003).

1.9 BIOLOGICAL ACTONS OF INTERLEUKIN-6

The pleiotropic actions of IL-6 modulate a variety of physiological events. Since its original description as a B cell differentiation factor, promoting differentiation of normal B cells into antibody producing cells (Hirano et al., 1986) its diverse effects on a range of different organs and cells have been revealed. Interleukin-6 has been reported to be involved in the induction of proliferation of hybridoma/plasmacytoma/myeloma cells, inhibition of proliferation in various cancer cell lines, induction of IL-2 production, cell growth, and cytotoxic T cell differentiation of T cells, induction of neuronal differentation (Kamimura et al., 2003), and growth/hypertrophy of skeletal muscle satellite cells (Serrano et al., 2008). Furthermore, IL-6 has been shown to have a protective effect on apoptosis in neutrophils (Kim et al., 1999b), and β-cells (Choi et al., 2004). Moreover, IL-6 is involved in regulating the immune system, the hematopoietic system, and in controlling the inflammatory response through proinflammatory and anti-inflammatory effects. Finally, IL-6 also acts on the nervous system, endocrine system, and has effects on bone metabolism, as well as other tissues and organs (Kamimura et al., 2003).

1.9.1 Interleukin-6 and type 2 diabetes

Interleukin-6 was initially described as a proinflammatroy cytokine mainly affecting the immune system, however, this classification of IL-6 has proven to be too simplistic. In the adaptive and innate immune systems, IL-6 is involved in both amplification and protection against inflammation. Thus, inappropriate regulation of IL-6 may play a direct protective, or deleterious role in both antigen- specific immune-mediated diseases and in diseases where IL-6 or other inflammatory factors cause a low-grade inflammation, as seen in obesity and type 2 diabetes. Indeed, circulating IL-6 levels are elevated in obese and type 2 diabetic patients, and have been found to predict future risk of type 2 diabetes development (Spranger et al., 2003).

Studies investigating the association between IL-6 gene variants and T2DM have been contradictory, whereas the IL-6 receptor gene locus has been linked to T2DM in genome wide-scans for T2DM loci. The IL-6 receptor gene maps to a region with repeated linkage to T2DM. Genetic variants in the IL-6 receptor gene have been evaluated in four independent populations. The main focus has been two nonsynonymous exon 9 SNPs: IL-6 receptor+48867C>A (rs8192284) and IL-6 receptor+48947G>T (rs2228146) leading to an Asp358Ala and a Val385Ile polymorphism, respectively, in the mature IL-6 receptor (Hamid et al., 2004; Wang et al., 2005). The common Asp358 variant was associated with T2DM and conferred a significant risk to T2DM in this population (Hamid et al., 2004; Wang et al., 2005). Thus, there is some evidence pointing to a role of the IL-6 receptor Asp358 polymorphism in conferring risk to T2DM, however, the pathogenetic mechanism is not known. Obesity and/or insulin resistance do not seem to be the explanation; the Ala358 variant is associated with increased BMI in some populations (Wolford et al., 2003) and not in others (Hamid et al., 2004; Wang et al., 2005). Further, the Ala358 polymorphism does not affect insulin sensitivity (Hamid et al., 2004; Wang et al., 2005). Point mutations involving codon 358 do not affect expression of the membrane-bound IL-6 receptor, but the Asp358 polymorphism may affect the levels of sIL-6 receptor as the IL-6 receptor is shedded by proteolytic cleavage in the Gln357/Asp358 juncture (Jones et al., 2001). Substitution of aspartate with glycine at amino acid position 358 reduces shedding to 25%, and Ala357/Ala358 double-point mutations attenuates shedding by 66% *in vitro*. Thus, carriers of the Ala358 allele may have reduced circulating levels of sIL-6 receptor due to impaired cleavage, and this may in turn result in reduced IL-6 signalling in tissues only expressing gp130, and potentially increased signalling in cells expressing the membrane bound IL-6 receptor.

1.9.2 Interleukin-6 effects on pancreatic islet function and survival

Apoptosis is the main cause of β-cell death in T1DM (Eizirik and Mandrup-Poulsen, 2001) and of reduced β-cell mass in type 2 diabetes (Butler et al., 2003a). *In vitro* studies of islets and β-cells have not demonstrated an apoptotic effect of IL-6 alone. Indeed, IL-6 has been shown to impair β-cell insulin secretion, but only in the presence of other pro-inflamamtory cytokines such as IL-1β (Eizirik et al., 1994). Additionally, an *in vitro* anti-apoptotic effect of IL-6 on islets and the MIN6 β-cell line has been reported (Choi et al., 2004). Overexpression of IL-6 in islets did not cause any changes in apoptosis (Campbell et al., 1994; DiCosmo et al., 1994). Hence, the role of IL-6 in regulating β-cell apoptosis is not clear, and further studies to elucidate the specific effects of IL-6 not only on β-cell, however also on α-cell survival are warranted.

In addition to a decreased β-cell mass due to increased apoptosis, insulin production and secretion are impaired in T2DM (Reaven et al., 1988). *In vitro* studies on human and rodent islets investigating the effect of IL-6 on insulin production and release have not demonstrated consistent effects, however most studies show that IL-6 inhibits glucose stimulated insulin secretion from rodent islets (Kristiansen and Mandrup-Poulsen, 2005), whereas no effect of IL-6 alone was observed in

human islets (Eizirik et al., 1994). These inconsistent results may partly be explained by the wide range of IL-6 concentrations used, as well as differences in incubation time. Impaired β-cell function in IL-6 transgenic mice has not been reported, and IL-6 knockout mice have identical fasting insulin levels when compared with IL-6 $^{+/+}$ littermates (Di Gregorio et al., 2004) arguing against an independent *in vivo* effect of IL-6 on β-cell function. There was no effect on plasma insulin levels in healthy males challenged with IL-6, but an increase in plasma glucagon was observed after 1 hour (Tsigos et al., 1997; van Hall et al., 2003). Thus, an acute increase in IL-6 does not affect β-cell function in humans; a finding supported by studies in rodents (Kim et al., 2004).

Whether IL-6 is able to act directly on β-cells and/or α-cells has never been reported. Indeed, to increase our understanding of already reported findings, as well as future findings, studies investigating the expression of the IL-6 receptor and gp130 in pancreatic β-cells and α-cells are warranted.

1.9.3 Interleukin-6 effects on insulin action in skeletal muscle

The role of IL-6 in insulin action and peripheral insulin resistance is controversial and has led to the ongoing debate whether IL-6 is a "good or bad guy" (Mooney, 2007) (Pedersen and Febbraio, 2007). Skeletal muscle is the largest insulin sensitive tissue contributing to >75% of insulin stimulated glucose disposal in healthy individuals (Saltiel and Kahn, 2001). Whether IL-6 acts as an insulin sensitizer or desensitizer in skeletal muscle is debated, however the majority of evidence points towards an insulin sensitizing effect of IL-6. Various *in vivo* and *in vitro* studies have shown that IL-6 enhances insulin-dependent glycogen synthesis and glucose uptake in skeletal muscle (Carey et al., 2006; Weigert et al., 2005; Pedersen et al., 2004). Weigert et al. 2005 showed that IL-6 directly interacts with insulin signal transduction by induction of serine phosphorylation of IRS-1 in various muscle cell lines and in muscle tissue of mice. Furthermore, the authors show that the insulin sensitizing effect of IL-6 on glycogen synthesis in human skeletal muscle cells is through phosphorylation of PKB.

1.9.4 Interleukin-6 effects on insulin action in adipocytes

Interleukin-6 has adverse effects on insulin action in adipose tissue as demonstrated in adipocyte cell line studies. More specifically, IL-6 decreased IRS-1 protein expression, insulin stimulated tyrosine phosphorylation, reduced insulin stimulated glucose uptake (Rotter V 2003 JBC), and inhibited insulin induced activation of PKB, and ERK-1/2. It further suppressed insulin induced lipogenesis and glucose transport by reducing expression of GLUT4 (Lagathu et al., 2003). However, short-term IL-6 treatment has also been shown to enhance glucose transport, and the effects are additive to insulin stimulated glucose transport (Stouthard et al., 1996). *In vivo* infusion of IL-6 in a physiological concentration increased subcutaneous adipose tissue glucose uptake in humans (Lyngso

et al., 2002) arguing against IL-6 as an insulin resistance-inducing agent in adipocytes, but independent confirmation is warranted. Studies investigating the role of IL-6 in insulin resistance in adipocytes *in vitro* and *in vivo* have mainly looked at short term effects of high levels of IL-6. This is likely to elicit a different response (Fasshauer et al., 2003) compared with the long-term low-grade IL-6 stimulation seen in individuals developing insulin resistance (Kristiansen and Mandrup-Poulsen, 2005).

1.9.5 Interleukin-6 effects on insulin action in hepatocytes

Studies investigating the effect of IL-6 on insulin action in hepatocytes display consistent results (Kristiansen and Mandrup-Poulsen, 2005). Rodent *in vivo* and *in vitro* studies, and *in vitro* studies on the human hepatocarcinoma HepG2 cell line indicate that IL-6 signalling in hepatocytes is mainly directed via the JAK/STAT pathway leading to STAT3 phosphorylation, SOCS3 transcription, inhibition of 1) insulin receptor autophosphorylation and 2) tyrosine phosphorylation of IRS-1 and IRS-2, decreased glycogen storage due to decreased gluconeogenesis and increased glycogenolysis. Collectively these studies provide strong evidence for the ability of IL-6 to reduce insulin sensitivity in hepatocytes by hampering insulin signalling. However, the regulation of hepatic glucose production by IL-6 is an ongoing debate. While IL-6 infusion during exercise has been shown to increase endogenous glucose production (Febbraio et al., 2004) and a single dose of IL-6 increased fasting blood glucose levels in resting subjects (Tsigos et al., 1997), other studies failed to show enhanced glucose output from the liver (Steensberg et al., 2003). Recently, the crosstalk of IL-6 and insulin in the regulation of hepatic gluconeogenesis received a novel fascinating aspect, at least in rodents. Central effects of insulin leading to suppression of glucose production are mediated by IL-6 synthesis in hepatic non-parenchymal cells (Inoue et al., 2006).

1.9.6 Interleukin-6 effects on the central nervous system

The recent interest in the actions of IL-6 on cerebral centers involved in the regulation of energy expenditure is based on the observation that IL-6 knockout mice develop mature onset of obesity, impaired glucose tolerance, and hyperglycemia (Wallenius et al., 2002). Obesity in IL-6 knockout mice was partly reversed by long-term IL-6 replacement administered centrally (Wallenius et al., 2002), and more strikingly intracerebroventricular, but not intraperitoneal IL-6 injection increased energy expenditure without changing the respiratory exchange ratio, suggesting that the anti-obesity effect of IL-6 is mainly exerted at the level of the CNS. Single peripheral administration of IL-6 also increases energy expenditure in humans (Wallenius et al., 2003). Repeated daily intracerebroventricular injections of IL-6 for 14 days in mice and adenoviral IL-6 expression in the hypothalamus for 5 weeks in rats led to reduction in body weight and reduced fat mass compared with control animals without significant reduction in food intake per body weight. Interleukin-6 levels in

cerebrospinal fluid correlate negatively with total body fat in obese humans and the cerebrospinal fluid levels are of the same magnitude as in serum, suggesting that cerebrospinal fluid IL-6 is at least in part regulated independently of serum IL-6, possibly by local production in the brain (Wallenius et al., 2003). A further indicator of a physiological role for IL-6 in the brain is the induction of locally produced IL-6 in the brain in response to exercise (Nybo et al., 2002).

1.10 THESIS INVESTIGATION

There exists a potential physiological role for IL-6 in maintaining glucose homeostasis. With respect to pathophysiology, circulating IL-6 levels are increased in obese individuals and patients with T2DM (Herder et al., 2005), and increased plasma levels of IL-6 predict disease development (Spranger et al., 2003). Furthermore, IL-6 receptor polymorphisms map to a region of repeated linkage to T2DM (Hamid et al., 2004; Wang et al., 2005). Thus, considering the limited and confounding knowledge of IL-6 actions on the pancreatic islet, it is of great importance to elucidate whether IL-6 has direct effects on pancreatic islet cells, and whether these effects are of significance in regulating glucose homeostasis and in the development and progression of type 2 diabetes. Elucidating this may lead to knowledge on how to apply strategies for therapeutic intervention in the treatment of diabetes.

The present thesis investigation was undertaken to elucidate the effects of IL-6 on human and rodent (mouse and rat) pancreatic islets *in vitro*, and moreover to study the role of IL-6 in regulating glucose homeostasis and islet cell mass in the mouse *in vivo*. The following hypotheses were tested:

Hypothesis 1: The IL-6 receptor is expressed by islet endocrine cells, and IL-6 directly regulates α-cell and/or β-cell secretory function and survival.

Hypothesis 2: Interleukin-6 is involved in maintaining glucose homeostasis and islet endocrine cell mass during the development of obesity induced by HF diet feeding.

CHAPTER 2: METHODOLOGY

2.1 REAGENTS, TISSUE CULTURE DISPOSABLES, AND ANTIBODIES

All chemicals, of reagent or molecular biology grade were from Invitrogen Corporation (Basel, Switzerland), R&D Systems (Minneapolis, Minnesota, USA), Sigma Tau (Rome, Italy), Sigma-Aldrich Corporation (Buchs, Switzerland), and Roche (Basel, Switzerland).

Tissue culture disposables were from Techno Plastic Products (Trasadingen, Switzerland). Extracellular matrix coated (ECM) culture dishes, derived from bovine corneal endothelial cells were from Novamed (Jerusalem, Israel), and 804G-ECM culture dishes were prepared with 804G cells as previously described (Hammar et al., 2005). Briefly, 804G cells, a rat bladder carcinoma cell line that produces ECM rich in laminin-5 (epiligrin) and moreover contains fibronectin (Hammar et al., 2004), both have been show to induce attachment and spreading of many epithelial cell types, including pancreatic islet β-cells (Hayek et al., 1995).

Primary antibodies used for immunostaining of pancreatic tissue sections and islet cells *in vitro* were: guinea-pig anti-insulin (#A0564, Dako, Glostrup, Denmark), rabbit anti-glucagon (#A0565, Dako), guinea-pig anti-glucagon (#4031-01F, Linco Research, St. Charles, New Brunswick, USA), rabbit anti-IL-6 receptor (#sc-660, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), rabbit anti-gp130 (#sc-656, Santa Cruz), rabbit anti-phospho-Stat3 (Tyr705) (#9131, Cell Signaling Technology Inc., Danvers, Massachusetts, USA), rabbit anti-Stat3 (#9132, Cell Signaling), rabbit anti pERK1/2 Map Kinase (Thr202/Tyr204) (#9101, Cell Signaling), rabbit anti-phospho-JAK2 (#07-123, Cell Signaling), rabbit anti-prohormone convertase (P/C) 1/3 (kindly provided by Donald F. Steiner) (Smeekens et al., 1992). A rabbit anti-Ki67 (#RM-9106, Lab Vision Inc., Fremont, California, USA) was used for pancreatic tissue sections, and a mouse anti-Ki67 (#08-0156, Zymed Laboratories Inc., San Francisco, California, USA) was used for islet cells *in vitro*. 5-Bromo-2-deoxyuridine (BrdU) incorporation was detected with a mouse anti-BrdU antibody (BrdU Labeling and Detection Kit II, #1299964, Roche). Apoptosis in pancreatic tissue sections was detected using the ApopTag® Peroxidase *in situ* Detection Kit (#S7100, Q-BIOgene MP Biomedicals, Illkirch, France), and apoptotic islet cells *in vitro* were visualized using the *In situ* Cell Death Detection Kit, AP (#11 684 809 001, Roche).

Primary antibodies used for electron microscopy and for staining semithin sections from human islets were, mouse anti-glucagon (#G-2654, Sigma), rabbit anti-IL-6 (#PR627, Pierce, Rockford, Illinois, USA), and guinea-pig anti-insulin (#A564, DAKO).

Specific sources of chemicals not mentioned are indicated in brackets by the company name in the following sections describing the experimental methodology.

2.2 PANCREATIC ISLET ISOLATION

Human islets were isolated from pancreata of organ donors at the University of Geneva Medical Center in Geneva, Switzerland, and at INSERM ERIT-M 0106, in Lille, France. In all human islet preparations used, the islet purity was >70% islets, as assessed by dithizone staining (#43820, Sigma). Dithizone is a zinc chelator and stains insulin-containing cells bright red, hence enabling the distinction of islets from non islets (exocrine tissue), and consequently allows assessment of islet purity.

Mouse islets were isolated from C57BL/6J mice (Harlan, Horst, Netherlands) by collagenase (#4189, type 4, activity 201 u/mg, Worthington, Lakewood, New Jersey, USA,) digestion. Collagenase solution was made with 50 ml Hank's Buffered Salt Solution (HBSS, #24020, Invitrogen), 500 µl 1M HEPES (#15630-056, Invitrogen), 16.5 µl Dnase (#11284932001, Roche) (3.3 µg/ml), and collagenase 2 mg/ml final concentration). Collagenase solution (2 ml) was infused into the pancreatic duct, and other parts of the pancreas to inflate the pancreas with collagenase solution as best as possible. This was followed by a 30 minutes digestion in additional 2 ml collagenase solution (water bath 37°C). The digestion was stopped by adding quenching buffer (500 ml HBSS, 12 ml 1M HEPES, 2.5 g BSA (0.5% w/v; Bovine Serum Albumin, #A7888, Sigma). After centrifugation, washing, and filtration, Histopaque-1119 (#H8889, Sigma) was used to make a density gradient (1100 density: 20 ml Histopaque-1119 + 4 ml HBSS; 1080 density: 16 ml Histopaque-1119 + 8 ml HBSS; 1060 density: 12 ml Histopaque-1119 + 12 ml HBSS). Islets were separated from exocrine tissue by centrifugation, floating on the border of layers with 1100 and 1080 density. Thereafter, mouse islets were handpicked to ensure >90% islet purity.

Rat islets were isolated from male Wistar rats (150 - 250 g, 6 - 11 weeks; Janvier, Le Genest, France) by collagenase digestion (Worthington) followed by ficoll purification, using a modification of previously described procedures (Rouiller et al., 1990) (Sutton et al., 1986).

2.3 PRIMARY SINGLE ISLET CELL CULTURE

To obtain single islet cells, mouse islets, either freshly isolated or already cultured on ECM coated dishes, were washed twice in Mg^{2+} and Ca^{2+}-free phosphate-buffered saline (PBS: 80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 17.8 g Na_2HPO_4 $2H_2O$, 2.5 g KH_2PO_4 in 10 litre H_2O + 0.5 mM EDTA (#15575-020, Invitrogen)). Thereafter, islets were incubated for 6 minutes at 37°C in 300 µl trypsin-EDTA (#25 300-054, Invitrogen) and 200 µl Mg^{2+} and Ca^{2+}-free PBS + 0.5 mM EDTA per 30 - 50 islets/dish, or in 600 µl trypsin-EDTA and 900 µl Mg^{2+} and Ca^{2+}-free PBS + 0.5 mM EDTA per 700 islets (in 15 ml Falcon tube). To ensure single cells, islets were mechanically dispersed (by pipetting), and culture media was added to stop trypsinization. Cells were centrifuged (1000 rpm for 3 minutes), resuspended in culture media, and plated back on ECM coated dishes (4000 cells/dish) for proliferation studies, or centrifuged using a Cytospin 4 (Thermo Fisher Scientific Inc., Waltham,

Massachusetts, USA) onto slides (for apoptosis studies). For some studies, islets were dispersed in to single cells 24 hours after isolation, and cultured as single cells (4000 cells/dish).

2.4 FLUORESCENT ACTIVATED CELL SORTING OF PRIMARY SINGLE ISLET CELLS

Rat islet α-cells and β-cells were isolated from male Wistar rats. Islets were rinsed three times with Mg^{2+} and Ca^{2+}-free PBS and resuspended in 1.5 ml of the same buffer containing trypsin-EDTA, and digested for 6 minutes at 37°C (with occasional pipetting). Digestion was stopped by adding 10 ml ice cold Krebs-Ringer bicarbonate buffer (KRBB, 6.7 g NaCl, 0.35 g KCl, 0.38 g $CaCl_2$ $2H_2O$, 0.16 g KH_2PO_4, 0.29 g $MgSO_4$ $7H_2O$, in 1000 ml H_2O), pH 7.4, containing 0.5% BSA, 2.8 mM glucose, and 10 mM HEPES. β-Cells were then separated from non-β-cells by autofluoresence, using a fluorescence-activated cell sorter (FACS), (FACStar-Plus cell sorter, Becton Dickinson, Sunnyvale, California, USA), as previously described (Rouiller et al., 1990). α-Cell and β-cell purity was approximately 90% as assessed by glucagon and insulin staining respectively.

2.5 PRIMARY CELL CULTURE

For all *in vitro* studies, human and mouse islets were cultured on ECM coated dishes. Human islets were cultured in CMRL-1066 medium (#21530, Invitrogen) containing 5.5 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (#10500-064, Invitrogen). Mouse islets were cultured in RPMI-1640 medium (#31870, Invitrogen) containing 11 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml fungisone, 10 µg/ml gentamycin, and 10% FCS (all from Invitrogen), hereafter referred to as culture medium. In experiments where chronic glucagon release was assessed, 3 µg/ml aprotinin (#A3428, Sigma) was added to the culture medium, to avoid glucagon degradation (Andersson et al. 1981 In vitro Cellular and Developmental Biology – Plant).

FACS sorted rat α-cells and β-cells were cultured on 804G-ECM in Dulbecco's Modified Eagle's Medium (DMEM) (#11966, Invitrogen) containing 10% FCS, 11.2 mM glucose, 110 µg/ml sodium pyruvate (Invitrogen), supplemented with antibiotics. Aliquots of $3x10^5$ cells were seeded in non-adherent 100 mm diameter Petri dishes containing 9 ml medium. Cells were incubated for 20 hours at 37°C to allow full recovery of any cell surface molecules that may have been lost or damaged during islet isolation or cell purification. After recovery, cells were resuspended at a concentration of $4x10^5$ cells/ml, and aliquots of 50 µl were plated as droplets on 3 cm plastic dishes coated with 804G-ECM.

2.6 TOTAL DNA AND RNA EXTRACTION, AND PCR

In experiments where RNA was extracted from mouse and human islets, islets were plated on ECM coated dishes (80 islets/dish), and left overnight to attach. When attached, culture media was

changed and conditions added. RNA from FACS sorted α-cells and β-cells was extracted from freshly isolated islets, and from INS-1 β-cells 832 - 13, at passage 46 - 52. Total mouse and human islet RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel GmbH, Oensingen, Switzerland). Briefly, extraction involved an on the column DNAase digestion and was carried out according to the manufacturer's instructions. To increase RNA yield, the elution of the column was done by incubating the spin columns with the elution buffer for 15 minutes at room temperature prior to centrifugation, and by running the elute twice over the column. Total RNA was assessed using the Nanodrop (Thermo Fisher Scientific, Delaware, USA) and 25 ng/µl RNA was reverse transcribed using the SuperscriptTM II Rnase H^- reverse transcriptase kit (Invitrogen Ltd., Basel, Switzerland) following the instructions of the manufacturers. RNA was primed with random hexamers (Microsynth, Balgach, Switzerland), and reaction was carried out at 42°C for 50 minutes followed by 15 minutes at 70°C. Total RNA from FACS sorted islet cells was extracted as previously described (Pipeleers et al., 1985a).

For Polymerase Chain Reaction (PCR), human and mouse primers for the IL-6 receptor and gp130 were purchased from Microsynth. Human primers: IL-6 receptor forward (5'→3') GAC AAT GCC ACT GTT CAC TG, reverse (5'→3') GCT AAC TGG CAG GAG AAC TT. Gp130 forward (5'→3') ACA GAA CAG CAT CCA GTG TC, reverse (5'→3') AAT CTG GCT CCA AGT TGA GG. Tubulin forward (5'→3') AGA GTC GCG CTG TAA GAA GC, tubulin reverse (5'→3') TGG TCT TGT CAC TTG GCA TC. The PCR was performed under the following conditions: 95°C for 15 minutes, followed by 44 cycles of 94°C for 1 minute, 68°C and 70°C for 1 min (for IL-6 receptor and gp130 respectively), and 72°C for 10 minute.

Mouse primers: IL-6 receptor forward (5'→3') AGC TTG GTT CCG ATT TCC TT, reverse (5'→3') TTC GCC TGA AGT CCT GAG AT. Gp130 forward (5'→3') GCA GCA GGT TTC AGA TCA CA, reverse (5'→3') TCA GGA GCC AGT CCT TCA CT. GAPDH forward (5'→3') GTG GCA GTG ATG GCA TGG AC, GAPDH reverse (5'→3') CAG CAC CAG TGG ATG CAG GG. The PCR was performed under the following conditions: 95°C for 15 minutes, followed by 45 cycles of 94°C for 1 minute, 55°C and 58°C for 1 minute (for IL-6 receptor and gp130 respectively), and 72°C for 10 minutes.

Samples (10 µl cDNA) were run on a 2% agarose gel (100 ml Tris-acetate-EDTA buffer (TAE), 2 g agarose, and 5 µl ethidium bromide (EtBr)).

For quantitative real-time PCR (RT-PCR) the following commercially available primers were purchased from Applied Biosystems (Foster City, USA): 18S ribosomal RNA (rRNA) (#Hs99999901_s1), human glucagon (#Hs00174967_m1), human insulin (#Hs00355773_m1), rat IL-6 receptor (#Rn_00566707_m1), rat gp130 (#Rn_01489669_m1), mouse bcl-2 (#Mm00477631_m1), mouse c-myc (#Mm00487803_m1), mouse p27 (Cdkn1b) (#Mm00438167_g1), mouse cyclin D1 (#Mm00432360_m1), mouse D2 (#Mm00438072_m1), and mouse D3 (#Mm01273583_m1). These assays were carried out using commercial TaqMan gene expression assays and the RT-PCR system ABI 7000 (Applied Biosystems) according to manufacturers protocol. The RT-PCR was performed

using the following thermal profile: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 1 minute at 60°C (default settings).

Changes in mRNA expression were calculated using difference of cycle threshold (Ct) corrected for different amount of cDNA input, with 18S RNA serving as reference. A mixture of cDNA and H_2O (1:10 dilution) was combined with 2x Universal PCR Master Mix and 20x primerprobe (1:1.2 dilution). 18S samples were diluted further (1:10), and all samples were run in triplicate.

DNA from mouse tails was extracted for genotyping using a DNA extraction Kit (Strategene, La Jolla, USA) following the manufacturers protocol. Primers for genotyping the IL-6 knockout mouse were: IL-6 forward (5'→3') TTC CAT CCA GTT GCC TTC TTG G, IL-6 reverse (5'→3') TTC TCA TTT CCA CGA TTT CCC AG, and IL-6 neo CCG GAG AAC CTG CGT GCA ATC C, as recommended by Jackson Laboratories (Bar Harbour, Maine, USA).

2.7 GENE ARRAY

The gene array was performed in collaboration with the laboratory of Dr. Frans C. Schuit at the Department of Molecular Cell Biology, Gene Expression Unit, Katholieke University in Leuven, Belgium.

Total RNA from mouse and rat pancreatic islets and acini was extracted using Absolutely RNA Microprep (Stratagene, La Jolla, California, USA), while RNA from whole tissues and INS-1 β-cells were extracted using TRIzol (Gibco), followed by a cleanup with RNeasy columns (Qiagen, Hombrecthikon, Switzerland). RNA was reverse transcribed into cDNA (SuperScript Choice System Invitrogen, using oligo-dT primers and a T7 RNA polymerase promoter site); cDNA was *in vitro* transcribed and biotin-labelled (Affymetrix IVT labelling kit.) Quantity and quality of total RNA and cRNA profiles of all RNA preparations were analysed, respectively using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent), the latter yielding distinct cRNA peaks corresponding to proinsulin and zymogen cRNA species for islets and acini, respectively. mRNA quantification was performed using Affymetrix mouse 430 2.0 and Affymetrix rat 230 2.0 expression microarrays. The overnight biotin-labelled cRNA was fragmented during 35 min at 94°C and hybridized during 16 hours at 45°C, followed by washing/staining in a Fluidics Station (Affymetrix) and scanning using a 3000 GeneScanner. Raw data were analyzed using GCOS software. Signal intensities were scaled using the global scaling method taking 150 as target intensity value.

2.8 PROTEIN EXTRACTION AND WESTERN BLOT

For Western blotting experiments, mouse and human islets were plated on ECM coated dishes (100 islets/dish) and left overnight to attach. When attached, culture medium was changed and

conditions added. Afterwards, islets were washed in PBS, and protein extracted with 30 µl lysis buffer (20 mM Tris acetate (pH 7.0), 0.27 mol/l sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1% Triton X-100, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate), containing 1 mM dithiothreitol, 1 mM benzamidine, 4 µg/ml leupeptin, and 100 mM PMSF (added before use) per 100 islets. Samples were left on ice for 30 minutes, followed by sonication (30 seconds), and centrifugation (12 000 rpm for 30 minutes at 4°C). Protein content was quantified using the BCA kit (Pierce, Rockford, Illinois, USA) in order to ensure equal loading of gels for subsequent Western blotting. Samples were stored at –80°C until assayed.

Equivalent amounts of protein lysate (20 – 50 µg) were mixed with SDS sample buffer, boiled for 10 minutes, and subjected to 10% SDS-PAGE gel. Proteins were electrically transferred to nitrocellulose filter membranes. Blocking of non-specific protein binding was done by incubating filter membranes in blocking buffer (1xTBS (pH 7.6), 0.1% Tween-20, and 5% non-fat dry milk) for 1 hour. After washing in TBS-T (1xTBS (3.03 g Tris-HCl, 8.7 g NaCl in 1 litre H_2O; pH 7.6) and 0.1% Tween-20), filters were incubated at 4°C overnight with the following phosphospecific antibodies, pStat3 (Tyr705, 1:1000), pERK1/2 Map Kinase (Thr202/Tyr204, 1:1000), pJAK2 (1:1000), IL-6 receptor (1:100), and total Stat3 (1:1000). Subsequently, filter membranes were washed in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (1:4000). Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signaling), and light emission was captured on X-ray film.

2.9 ASSESSMENT OF α-CELL FUNCTION *IN VITRO*

For acute glucose and arginine stimulated glucagon secretion, human islets (30 islets/dish) were plated on ECM coated dishes in quintuplicate, and left overnight to attach. When attached, culture media was changed and conditions added. Experiments with various length of treatment were designed so they ended at the same time, this was done so total culture time was similar between conditions. Prior to assessing acute glucagon secretion, islets were washed and preincubated for 30 minutes in KRBB containing 20 mM glucose, 0.5% BSA, 10 mM HEPES, and 3 µg/ml aprotinin, to achieve a similar basal level of glucagon secretion. After 30 minutes, KRBB was replaced by 1 ml KRBB containing either 20 mM glucose (basal), 2 mM glucose (stimulated), or 10 mM arginine in 2 mM glucose, and islets were incubated for 1 hour at 37°C. Due to the stimulatory effect of insulin on glucagon secretion (Gromada et al., 2007), it was necessary that separate islets were used for basal and stimulated conditions respectively. Islet glucagon was extracted with 0.18 M HCl in 70% ethanol for determination of glucagon content. Glucagon secretion and content was assayed by radioimmunoassay (RIA) (Linco Research), and radioactivity was counted with a gamma counter (Kontron Analytical MDA 312, Montigny le Bretonneux, France). Nonspecific binding was defined as that measured in the presence of I-125 glucagon tracer and absence of glucagon antibody, whereas specific binding was expressed as the percentage of total binding (%B/B_o). Non-linear regression analysis of competitive

binding curves followed algorithms included in the Prism 4.0 software package (Graphpad, San Diego, California, USA). Determination of the glucagon concentration in the unknown samples was done by interpolation of the reference curve.

2.10 ASSESSMENT OF β -CELL FUNCTION *IN VITRO*

For glucose stimulated insulin secretion (GSIS), human and mouse islets (20 islets/dish) were plated on ECM coated dishes in triplicate, and left overnight to attach before culture media was changed and conditions added. Prior to assessing β-cell function, islets on ECM were washed and preincubated for 30 minutes in KRBB containing 2.8 mM glucose and 0.5% BSA (Sigma) and 10 mM HEPES (Invitrogen) to achieve similar basal insulin secretion. Thereafter, KRBB was replaced by 1 ml KRBB containing 2.8 mM glucose for 1 hour (basal) followed by an additional 1 hour in KRBB 16.7 mM glucose (stimulated). Islet insulin was extracted with 0.18 M HCl in 70% ethanol at 4°C overnight for determination of insulin content. Secreted insulin and insulin content was assayed by RIA (CIS Biointernational, Gif-Sur-Yvette, France), and radioactivity counted with a gamma counter (Kontron Analytical MDA 312). Unknown concentrations were calculated based on the principle of competitive binding between labeled insulin and insulin in the unknown samples for a fixed and limited number of antibody binding sites bound to the tube (coated tubes). After incubation, the unbound tracer was washed away, and the amount of labeled insulin bound to the antibody could be determined based on the inverse relationship to the amount of unlabeled insulin present in the sample.

2.11 ASSESSMENT OF PRIMARY ISLET CELL PROLIFERATION *IN VITRO*

Proliferation in human and mouse islets was assessed by Ki67 immunostaining and BrdU incorporation. Islets (30 islets/dish) were plated in triplicate on ECM coated dishes, and left for 2 days to attach and spread, before culture media was changed and conditions added. In experiments assessing BrdU incorporation, a final concentration of 10 μM BrdU (Roche) was present during the entire treatment period. At the end of the experiments, human and mouse islets were fixed in 4% paraformaldehyde (4 g PFA, 90 ml dH_2O, 10 ml 10xPBS, 0.1 ml 1N NaOH, pH 7.3) for 30 minutes at room temperature, washed in PBS, followed by permeabilization with 0.5% Triton X-100 in PBS for 4 minutes at room temperature. Thereafter, islets were washed with PBS and incubated with anti-Ki67 (Zymed, ready to use) for 30 minutes at 37°C, or anti-BrdU (Roche) for 30 minutes, at 37°C, 1:10 dilution. Ki67 positive cells were visualised using the Histostain *Plus* Broad Spectrum (AEC) kit (#85-9943, Zymed), and BrdU positive cells with a donkey anti-mouse Cy3 conjugated secondary antibody (#715-165-150, Jackson Immunoresearch Laboratories, Inc, West Grove, Illinois, USA) for 30 min, at 37°C, 1:100). Ki67 and BrdU-positive cells were counted using a light and fluoresence microscope (Zeiss, Feldbach Switzerland), and results expressed as number of proliferating islet cells per islet.

In some experiments mouse islets were dispersed into single cells (at the end of the experiment), distributed back on ECM coated dishes and allowed to adhere before being co-stained for islet hormones. Here, antibodies against BrdU (similar conditions as above), insulin (Dako, 30 minutes, 37°C, 1:50), and glucagon (Dako, 30 minutes, 37°C, 1:50) were used. Dapi (Invitrogen) was used for nuclei staining (10 minutes, 37°C, 1:1000). Secondary antibodies for insulin and glucagon were a FITC labelled rabbit-anti guinea-pig (#61-4611, Zymed), and a FITC labelled donkey anti-rabbit (#711-095-152, both 30 minutes, 37°C, 1:50). BrdU positive α-cells (glucagon positive) and β-cells (insulin positive) were counted using a fluorescence microscope (Zeiss), and results expressed as percent of total number of glucagon and insulin positive cells respectively. Approximately 400 α-cells and 1200 β-cells were counted per condition.

Assessment of proliferation (BrdU incorporation) in FACS sorted rat α-cells and β-cells was done in collaboration with Dr. Eva Hammar, in the laboratory of Philippe Halban, Department of Genetic Medicine and Development in Geneva. FACS sorted rat α-cells and β-cells were cultured on 804G-ECM for 24 hours, thereafter culture media was changed and conditions added. At the end of the experiment, cells were fixed in 1% PFA for 1 hour at room temperature, followed by a 1 hour DNA denaturation step with 1.5 M HCl at room temperature. Subsequently, cells were permeabilized for 4 min with 0.5% Triton X-100 in PBS at room temperature. Then cells were incubated with a mixture of primary antibodies (BrdU, Insulin, glucagon), followed by incubation with fluorescently labeled secondary antibodies, and finally cells were incubated with dapi for nuclear staining (as described for single cell staining). BrdU positive α-cells (glucagon positive) and β-cells (insulin positive) were counted using a fluorescence microscope (Zeiss), and results expressed as percent of total number of glucagon and insulin positive cells respectively. Approximately 1500 and 3000 α-cells and β-cells were counted per condition respectively.

2.12 ASSESSMENT OF APOPTOSIS *IN VITRO*

For apoptosis studies human and mouse islets were plated on ECM coated dishes (30 islets/dish) and left for 2 days to attach and spread before culture media was changed and conditions added. At the end of the experiment the free 3'-OH strand breaks resulting from DNA degradation were detected by TUNEL staining (In Situ Cell Death Detection Kit, AP; Roche Molecular Biochemicals, Mannheim, Germany). After washing with PBS, islets on ECM were fixed in 4% PFA for 30 minutes at room temperature, washed in PBS, followed by permeabilization with 0.5% Triton X-100 for 4 minutes at room temperature. Islets were incubated for 1 hour at 37°C with a TUNEL reaction mixture, followed by 30 minutes incubation at 37°C in the presence of an Alkaline Phosphatase (AP) converter. Subsequently, islets were rinsed with TBS, and incubated for 10 minutes at room temperature with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma) for visualization. TUNEL positive cells were counted using a light microscope (Zeiss) and results expressed as TUNEL positive cells per islet.

In some experiments apoptosis was determined in mouse single islet cells. Here the end labelling of DNA was performed with fluorescein-dUTP (2-deoxyuridine 5-triphosphate), without an AP converter, and cells were double stained for insulin and glucagon (both Dako, 30 minutes, 37°C, 1:50). Fluorescently labeled secondary antibodies were used to visualize hormone staining. For glucagon a cy3 labelled donkey anti-rabbit (#711-165-152, Jackson Immunoresearch, 30 minutes, 37°C, 1:50) was used, and for insulin a rhodamine labelled donkey anti guinea-pig (#706-025-148, Jackson Immunoresearch, 30 minutes, 37°C, 1:50) was used.

2.13 ANIMALS, ANIMAL HOUSING, AND HIGH FAT DIET FEEDING

Guidelines for the use and care of laboratory animals at the University of Zurich were followed, and ethical approval was granted by the Zurich Cantonal Animal Experimentation Committee. For high fat (HF) diet studies 4 - 12 week old male C57BL/6J wild type and B6;129S2-*Il6*^{tm1Kopf}/J (IL-6 knockout) mice backcrossed for 11 generations and maintained on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA).

Animals on a HF diet were fed a hypercaloric diet manufactured by Research Diets (New Brunswick, New Jersey, USA). The HF diet contained 58, 26 and 16% calories from fat, carbohydrate and protein, respectively, and a total of 5.6 kcal/g, whereas the control diet was manufactured by Provimi Kliba AG (Kaiseraugst, Switzerland) and contained 29, 39 and 32% calories from fat, carbohydrate and protein, respectively, and a total of 2.8 kcal/g. Animals were housed (five mice per cage) at 22°C with a 12 hour light-dark cycle (lights on at 7:00 am) and allowed free access to water and chow. During the 8 and 18 weeks of HF diet feeding, weighing the food given and removed monitored weekly food intake.

2.14 GLUCOSE TOLERENCE TEST

For glucose tolerance testing wild type and IL-6 knockout mice were fasted overnight (12 hours, 8pm to 8am) and injected intraperitoneally (i.p) with 2 mg/g body weight 40% glucose (Pharmacy of Dr. G. Bichsel AG, Interlaken, Switzerland). Blood glucose was measured with a Freestyle glucometer (Abbott, Baar, Switzerland) at 15, 30, 60, 90 and 120 minutes after glucose administration. Blood samples (40 µl whole blood) for insulin measurements were obtained from tail tip bleedings using capillaries and blood was left on ice in eppendorf tubes containing 50 mM EDTA. Blood samples were centrifuged (8000 rpm, 8 min) to separate plasma from red blood cells, and insulin was assessed by Luminex (Millipore, Billerica, Massachusetts, USA).

2.15 INSULIN TOLERENCE TEST

For insulin tolerance testing wild type and IL-6 knockout mice were fasted for 3 hours, starting at 10 am, and injected intraperitoneally with 0.75 mU/g body weight recombinant human insulin (Novo Nordisk A/S, Bagsvaerd, Denmark). Blood glucose was measured with a Freestyle glucometer at 15, 30, 60, 90 and 120 minutes after glucose administration.

Insulin resistance was also estimated based on fasting (12 hours) plasma insulin and blood glucose levels, using the homeostasis model for assessment of insulin resistance (HOMA-IR). The equation used was: (Fasting insulin X fasting glucose) divided by 22.5 (Wallace et al., 2004).

2.16 CIRCULATING HORMONES

Plasma insulin, glucagon, leptin, GLP-1, IL-6, IL-1β, TNF- α, KC, and MCP-1 were measured using mouse endocrine and cytokine multiplex assays (Millipore).

2.17 IMMUNOHISTOCHEMISTRY

Pancreata were fixed in 4% formalin for 12 hours and embedded in paraffin. Tissue sections (4 μM) were deparaffinised with xylol (three times 2 minutes), rehydrated with 95, 80, 70% EtOH (5 minutes each), and left in methanol for 6 minutes. For antigen retrieval, tissue sections were incubated for 10 minutes in citrate buffer (Zymed) at 350 watt in the microwave oven.

For islet morphometry, sections were incubated with the primary antibodies against insulin using a guinea-pig anti-insulin antibody, and against glucagon using a rabbit anti-glucagon (both Dako, 30 minutese, 37°C, 1:50). Fluorescently labelled secondary antibodies were used for visualization. For insulin a FITC labelled rabbit anti guinea-pig (Zymed, 30 minutes, 37°C, 1:50) was used, and for glucagon a donkey anti-rabbit Cy3-conjugated antibody (Jackson Immunoresearch, 30 minutes, 37°C, 1:50) was used.

For detection of the IL-6 receptor and gp130, the following antibodies were used: polyclonal rabbit anti-IL-6 receptor antibody (Santa Cruz, 30 minutes, 37°C, 1:10), and polyclonal rabbit anti-gp130 antibody (Santa Cruz, 30 min, 37°C, 1:50), both detected with a donkey anti-rabbit cy3 conjugated secondary antibody (Jackson Immunoresearch, 30 minutes, 37°C, 1:50). These sections were co-stained with an insulin antibody (Dako), and the primary glucagon antibody was a guinea–pig anti-glucagon antibody (#4031-01F, Linco), detected with the rabbit anti–guinea pig FITC conjugated secondary antibody (Zymed, 30 minutes, 37°C, 1:50).

Specificity of the IL-6 receptor and gp130 antibody was tested using isotype controls, blocking peptides (#sc-660P, #sc-656P, Santa Cruz), and by Western blotting using recombinant IL-6 receptor protein (#1830-SR, R&D) and blocking peptides.

To assess proliferation *in vivo*, 3 sections per animal with a minimum of 200 μm separation, averaging 80 - 100 islets in total per mouse, were stained with a Ki67 monoclonal rabbit antibody (NeoMarkers/Labvision, 1:100). Ki67 was detected with a biotinylated donkey anti-rabbit antibody (Jackson Immunoresearch, 1:80). These sections were co-stained with a rabbit anti-glucagon antibody (Dako), detected by a prediluted secondary antibody and chromogenically detected via the Ventana alkaline phosphatase Fast Red Kit. Counterstaining was done with hematoxylin.

The number of Ki67 positive and glucagon positive or non-glucagon positive cells were counted and expressed as % proliferating α-cells or β-cells of total number of α-cells or β-cells. In some cases results were expressed as Ki67 positive cells per islet.

Apoptosis *in vivo* was determined by TUNEL staining (Q-BIOgene, MP Biomedicals Illkirch, France), following the manufacturer's instructions. The staining was performed with an automated stainer (Ventana Benchmark; Ventana, Tucson, AZ). Sections were permeabilized with proteinase K (20 μg/ml, 15 minutes) and endogenous peroxidases blocked with 3% H_2O_2. Sections were incubated with working-strength TdT enzyme (ApopTag kit S7100; Millipore). After rinsing sections with a stop buffer, the slides were covered by anti–digoxigenin peroxidase conjugate, rinsed, and then incubated with rabbit anti-sheep horseradish peroxidase antibody (30 min, 1:80). Detection was performed with DAB (Ventana), and hematoxylin was used for counterstaining. Sections were co-stained with an rabbit anti-glucagon antibody, Dako, 1:50), followed by a prediluted secondary antibody and chromogenically detected via the Ventana alkaline phosphatase Fast Red Kit. Counterstaining was done with hematoxylin. Three sections per animal with a minimum of 200 μm separation, averaging 80 - 100 islets in total per mouse were evaluated. Results were expressed as % TUNEL positive α-cells or β-cells of total number of α-cells or β-cells. In some cases results were expressed as TUNEL positive cells per islet.

2.18 ISLET MORPHOMETRY

Paraffin embedded pancreatic tissue sections from wild type and IL-6 knockout mice were stained for insulin and glucagon as described in the previous section. Pancreatic section area, and insulin and glucagon positive cell area were determined from 3 pancreatic sections per animal, at 200 μm intervals, averaging 80 - 100 islets in total per animal. Pictures of islets stained for insulin and glucagon were acquired with an AxioCam HR camera connected to an Axioplan 2 microscope (Zeiss), and AxioVision software (Carl Zeiss Image) with a 20x magnification (Zeiss Plan, neofluar) objective. Measurements of insulin and glucagon positive cell area were done using the Image J NIH (National Institute of Health) software. Briefly, pictures were converted to 8 bit images; threshold and brightness were adjusted and applied. On the black and white image, area of interest was circled manually and scale set to: distance in pixels=1, known distance 0.34 μm (for 20x magnification), resulting in a 2.941 pixel/μm conversion.

Pictures of whole pancreatic section area were obtained with a LEICA MZ 16A stereo microscope (Spectronic, Leeds, UK), equipped with different light sources, a high performance digital colour camera and a motorized focus control. A 1x lens, with a 0.57x magnification was used, resulting in a conversion 6.7 μm/pixel.

The fraction of total glucagon and insulin positive cell area/total section area was multiplied by pancreatic weight to determine α-cell and β-cell mass, respectively. Islet density was calculated by dividing total islet number by the pancreatic section area.

2.19 IMMUNOCYTOCHEMISTRY

Mouse islets were plated on ECM coated dishes and left for approximately 6 days to allow complete spreading to optimize staining. Islets were fixed in 4% PFA for 30 minutes at room temperature, permeabilized with 0.5% Triton X-100 and incubated with a polyclonal rabbit anti-IL-6 receptor antibody, or a polyclonal rabbit anti-gp130 antibody (both Santa Cruz, for 30 min at 37°C (1:20 and 1:50 respectively). Islets were co-stained with either a guinea-pig anti-insulin antibody (Dako, 30 minutes, 37°C, 1:50), or a guinea-pig anti-glucagon antibody (Linco, 30 minutes, 37°C, 1:50), and visualized with FITC labelled secondary antibodies (as already described in the section Immunohistochemistry).

In one experiment, mouse islets on ECM were stained with a rabbit anti phospho-STAT3 antibody (Cell Signalling, 30 minutes, 37°C, 1:50), and co-stained with insulin (Dako, 30 minutes, 37°C, 1:50). Secondary antibodies were, donkey anti-rabbit cy3 (Jackson Immunoresearch, 30 minutes, 37°C, 1:50) for phospho-STAT3, and for insulin a FITC labelled rabbit anti guinea-pig (Zymed, 30 minutes, 37°C, 1:50) was used.

2.20 ELECTRON MICROSCOPY

Human islets from 4 separate isolations were fixed by immersion in a fixation solution containing 2.5% PFA, 0.1% glutaraldehyde and 0.01% picric acid for 4 hours. Specimens were dehydrated in an ascending series of ethanol and embedded routinely in LR White (Polysciences, Warrington, Pennsylvania, USA). Serial semithin sections (1 μm) were cut using an Ultracut E (Reichert-Jung, Zurich, Switzerland) and transferred onto glass slides. Non-specific binding was reduced by treatment for 30 minutes with 10 mM gelatine in 10 ml PBS (pH 7.4) containing 0.2 g BSA. Three consecutive semithin sections were processed for immunohistochemistry. The first section was incubated with a mouse antibody against human glucagon (#G-2654, Sigma, 4°C, 18 hours, 1:100), the second with a rabbit antiserum against rat IL-6 (#PR 627, Pierce Endogen, San Diego, California, USA, 4°C, 18 hours, 1:100), and the third with a guinea pig antiserum against porcine insulin (#A564, DAKO, 4°C, 18 hours, 1:2'000) all in a humid chamber. After washes in PBS, the sections were incubated with the respective biotinylated goat anti-species IgGs (BioScience Products,

Emmenbrücke, Switzerland; Amersham Int., Little Chalfont, UK, 30 minutes, 1:100), followed by incubation with a streptavidin-gold-5-nm complex (Amersham, 1 hour, room temperature, 1:100). After repetitive wash in double-distilled water, the sections were incubated with the IntenSE^{TM} M silver enhancement kit (Amersham) for 10 minuets at room temperature. The sections were counterstained with methylene blue/azure. Zeiss microscope and Axiovision 3.1. software (Zeiss) was used for picture taking.

Ultrathin sections were cut at 90 nm, transferred onto nickel grids, treated with 50 mM gelatine in 10 ml PBS containing 0.2 g BSA and rinsed in PBS. Sections were first incubated with the rabbit IL-6 antiserum overnight (1:50) followed by incubation with biotinylated goat anti-rabbit IgG (BioScience Products, 1:100), and a streptavidin-gold-15 nm complex (Amersham, 1:100). Sections were air-dried and incubated with the mouse glucagon antibody (1:50) overnight visualized by a 5-nm-gold-conjugated goat anti-mouse IgG. Sections were counterstained with uranyl acetate for 4 min and photographed with a CM100 electron microscope (Philips, Eindhoven, Netherlands).

2.21 DATA ANALYSIS

All data are expressed as means S.E.M. with the number of individual experiments presented in the figure legend. All data were analysed using the nonlinear regression analysis program Prism (GraphPad, San Diego, California, USA), and significance was tested using Student's t-test and analysis of variance (ANOVA) with Dunnett's multiple comparison test, and Bonferroni's post-hoc test as indicated in the figure legends. Statistical significance was set at 5%. All *in vitro* experiments were done in at least triplicate, and considered as an n=1. All counts and morphometry analysis was done blinded.

CHAPTER 3: INTERLEUKIN-6 AND THE INTERLEUKIN-6 RECEPTOR IN PANCREATIC ISLETS

3.1 BACKGROUND

It has been known since 1989 that the pancreatic islet is able to produce IL-6 (Campbell et al., 1989), and more recent studies have added IL-1β and PANDER to the list of α-cell and β-cell derived cytokines (Maedler et al., 2002; Yang et al., 2005). Elevated glucose increases IL-1β and PANDER secretion, both of which are detrimental to β-cell function and survival (Maedler et al., 2002; Yang et al., 2005). Furthermore, mouse and human islets exposed to a nutritional overload (33.3 mM glucose and 0.5 mM palmitate) show increased production and secretion of inflammatory factors including IL-8, chemokine KC, granulocyte-colony stimulating factor (G-CSF), and MIP-1α (Ehses et al., 2007). In addition, isolated islets from two type 2 diabetic rat models, the Goto-Kakizaki (GK) rat, and the Zucker diabetic fatty (ZDF) rat, show increased levels of islet derived inflammatory factors (Li et al., 2006).

These findings have established the paradigm that pancreatic islets produce various cytokines and chemokines in response to metabolic stress induced by a nutritional overload, and support the notion that T2DM is an inflammatory disease, not only at the level of insulin target tissues, but also at the level of the pancreatic islet. Therefore, these findings provided the rationale for investigating whether pancreatic islet cells respond to a nutritional overload, by increasing the production of IL-6.

Gp130 is ubiquitously expressed (Febbraio, 2007), whereas the expression of the IL-6 receptor is restricted. Despite the fact that several studies have demonstrated IL-6 effects on β-cells, no single report exists describing the presence or absence of the IL-6 receptor in the pancreatic islet. Thus, whether the IL-6 receptor has a role in pancreatic islet physiology and pathophysiology is unknown. Interestingly, the IL-6 receptor gene maps to a region of repeated linkage to type 2 diabetes, and IL-6 receptor gene polymorphisms are associated with the disease (Hamid et al., 2004; Wang et al., 2005), two observations, which add further relevance to the question whether there is a role for the IL-6 receptor in the pancreatic islet.

The intracellular signalling pathways activated by IL-6/IL-6 receptor/gp130 have never been reported in islets; however, IL-6 induced signalling has been extensively studied in numerous other cell types (reviewed by (Kamimura et al., 2003). The IL-6 receptor is coupled to activation of two distinct signaling pathways the JAK/STAT pathway, and SHP-2/ERK/MAPK pathway, known to induce a variety of important biological outcomes involved in the regulation of cell growth, differentiation, and survival. Thus, our aim was to elucidate whether the IL-6 receptor is functionally expressed in pancreatic islets, and coupled to known signal transduction pathways.

3.2 RESULTS

3.2.1 Pancreatic islet derived interleukin-6 is regulated by a nutritional overload

Initial studies were performed to elucidate whether a nutritional overload, induced by elevated nutrients (33.3 mM glucose and 0.5 mM palmitate), regulates IL-6 release from islets *in vitro*. Mouse and human islets exposed to 33.3 mM glucose and 0.5 mM palmitate (16:0), alone or in combination for 48 hours showed impaired glucose stimulated insulin secretion (GSIS), and released significantly more IL-6 in the presence of 33.3 mM glucose and 0.5 mM palmitate in combination compared to control conditions (11.1 mM and 5.5 mM glucose for mouse and human islets respectively) (Figure 2A-C). Basal IL-6 release was approximately 40 and 400 pg/ml/20 islets in mouse and human islets respectively, and the combination of 33.3 mM glucose and 0.5 mM palmitate resulted in a more than 3-fold increase in IL-6 released. Given the potential importance of these *in vitro* findings, we next sought to determine whether a nutritional overload increased islet derived IL-6 *in vivo*. Eight weeks on HF diet caused impaired glucose tolerance as compared to chow fed mice (Figure 2D), and indeed, islets from C57BL/6J mice fed a HF diet for 8 weeks released 2-fold more IL-6 compared to islets isolated from chow fed mice when cultured for 48 hours *ex vivo* (Figure 2E).

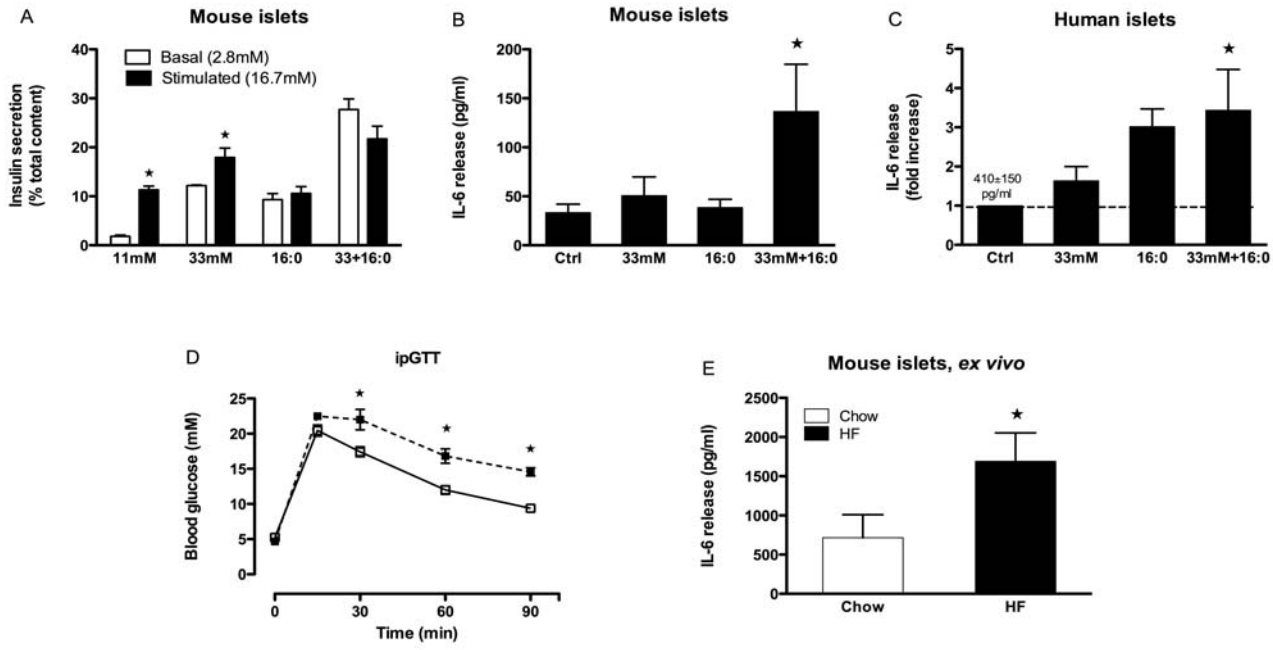


Figure 2: Nutritional overload induces IL-6 release from pancreatic islets *in vitro* and *in vivo*. Glucose stimulated insulin secretion in mouse islets (20 islets/well) treated for 48 hours on ECM coated dishes with high glucose (33.3 mM) and 0.5 mM palmitate (16:0) alone or in combination (33.3mM/16:0) (A, n=3-6). Interleukin-6 release from mouse (B, n=3-10) and human (C, n=7) islets treated for 48 hours on ECM coated dishes as indicated. The basal concentration of IL-6 release from human islets is provided on the graph, and results are expressed as fold increase. Intraperitoneal glucose tolerance test (2g/kg body weight) in mice after 8 weeks on chow (open squares) or HF diet (closed squares) (D, n=5). Interleukin-6 release from islets (20 islets/well) from mice fed a HF diet for 8 weeks (E, n=5); islets were cultured for 48 hours, and IL-6 was assessed by Luminex technology. * $p<0.05$ by Student's t-test (A, D, E versus open bars) or ANOVA with Dunnett's post-hoc test (B and C versus control).

Subsequent experiments were targeted at elucidating the specific cellular source of IL-6. These experiments were performed in non-treated human islets. For this, semithin sections (1µm) of human islets were generated and stained with antibodies directed against IL-6, glucagon, and insulin. This revealed IL-6 co-localization with glucagon positive α-cells (Figure 3, panel 2&3), a finding supported by EM, which further localized IL-6 to glucagon positive α-cell granules in human islets (Figure 3, panel 4). These experiments also revealed that not all glucagon positive α-cells were IL-6 positive supporting the specificity of the IL-6 antibody used.

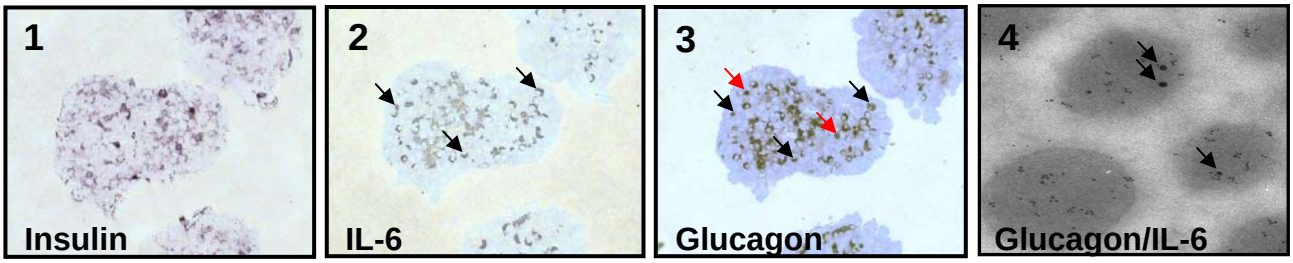


Figure 3: Interleukin-6 is localized to the glucagon positive α-cell granule in human islets. Images of immunostained semithin human islet sections (panel 1-3) stained for insulin (panel 1), IL-6 (panel 2) and glucagon (panel 3). Red arrows on panel 3 indicate glucagon positive cells that are not IL-6 positive. Black arrows on panel 2 and 3 indicate glucagon and IL-6 positive cells. Panel 4, EM image of secretory granules within an α-cell double gold-immunolabelled for glucagon (small particles) and IL-6 (large particles, black arrows) localizing IL-6 to glucagon containing granules (n=4).

Thus, the islet inflammatory stress response induced by a nutritional overload causes increased IL-6 release from pancreatic islets; and within the islets, IL-6 is localized to the α-cell glucagon granules.

3.2.2 Localization of the interleukin-6 receptor within the pancreatic islet

Based on the fact that IL-6 receptor expression in the pancreatic islet has never been reported, we took several approaches to elucidate its presence in the islet. In collaboration with the laboratory of Dr. Frans C. Schuit at the University in Leuven (Belgium), we conducted a tissue-expression profile of the IL-6 receptor and its signal transducing protein, gp130, in rodents: both mouse and rat. In mice, IL-6 receptor mRNA was highly expressed in the pancreatic islet compared to other tissues (Figure 4A), and the ubiquitously expressed gp130 was also detected in the pancreatic mouse islet (Figure 4C). This high islet cell expression of the IL-6 receptor was confirmed by comparison of various rat tissues (Figure 4B), and furthermore, rat α-cells showed a higher level of IL-6 receptor transcript compared to β-cells, or the rat INS-1 β-cell line (Figure 4B). Gp130 was also expressed at elevated levels in rat islet cells compared to other tissues (Figure 4D).

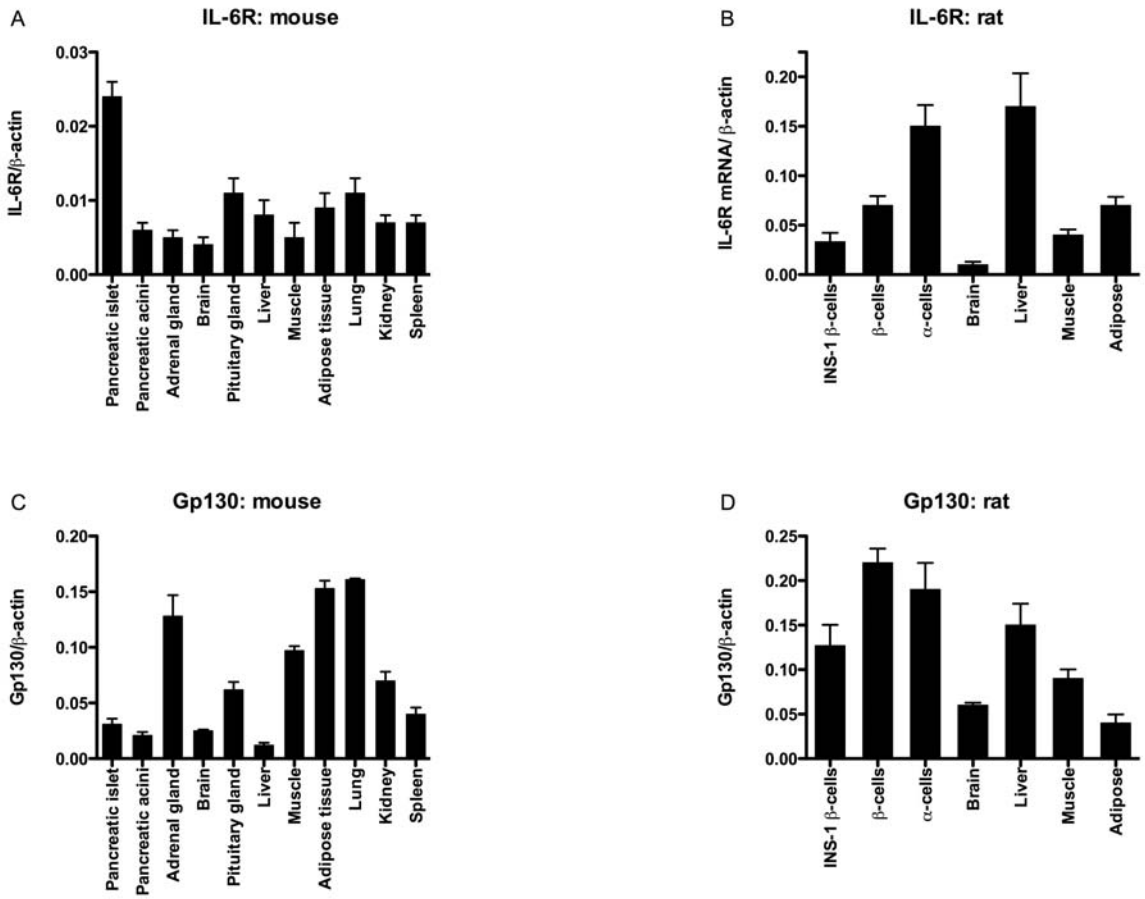


Figure 4: Interleukin-6 receptor (IL-6R) mRNA is highly expressed in the pancreatic islet. Tissue expression profile of the IL-6 receptor (A,B) and gp130 (C,D) in mouse (A,C) and rat tissues (B,D) determined by Affymetrix gene array (n=3-5). Note the elevated pancreatic islet mRNA expression of the IL-6 receptor in the mouse (A).

Several experiments were conducted in order to support the gene array data. Classical PCR on isolated mouse and human islets confirmed IL-6 receptor and gp130 receptor expression (Figure 5A), and quantitative real time PCR on mRNA from 90% pure FACS-sorted rat α-cells and β-cells (based on glucagon and insulin staining respectively) supported the gene array data insomuch as a higher level of IL-6 receptor transcript was found in the α-cell enriched fraction (Figure 5B). Gp130 receptor mRNA expression was not specifically elevated in rat α-cells (Figure 5C). Interestingly, comparison of IL-6 receptor mRNA expression in isolated islets from fed versus 18 hour fasted mice indicated a consistent upregulation of the receptor during fasting (Figure 5D).

Western blot analysis confirmed protein expression of the IL-6 receptor in mouse islets and in FACS sorted rat α-cells and β-cells. However, the western blot did not confirm elevated protein expression specifically in the α-cell (Figure 6A). Interestingly, co-staining of mouse pancreatic tissue sections and islets *in vitro* with antibodies against IL-6 receptor, glucagon, and insulin, localized the IL-6 receptor within the pancreatic islet to the α-cell (Figure 6B). Specificity of the antibody was confirmed using isotype controls and by absorption tests for immunohistochemistry (IL-6 receptor) (Figure 6B panel 7-8) and western blot analysis.

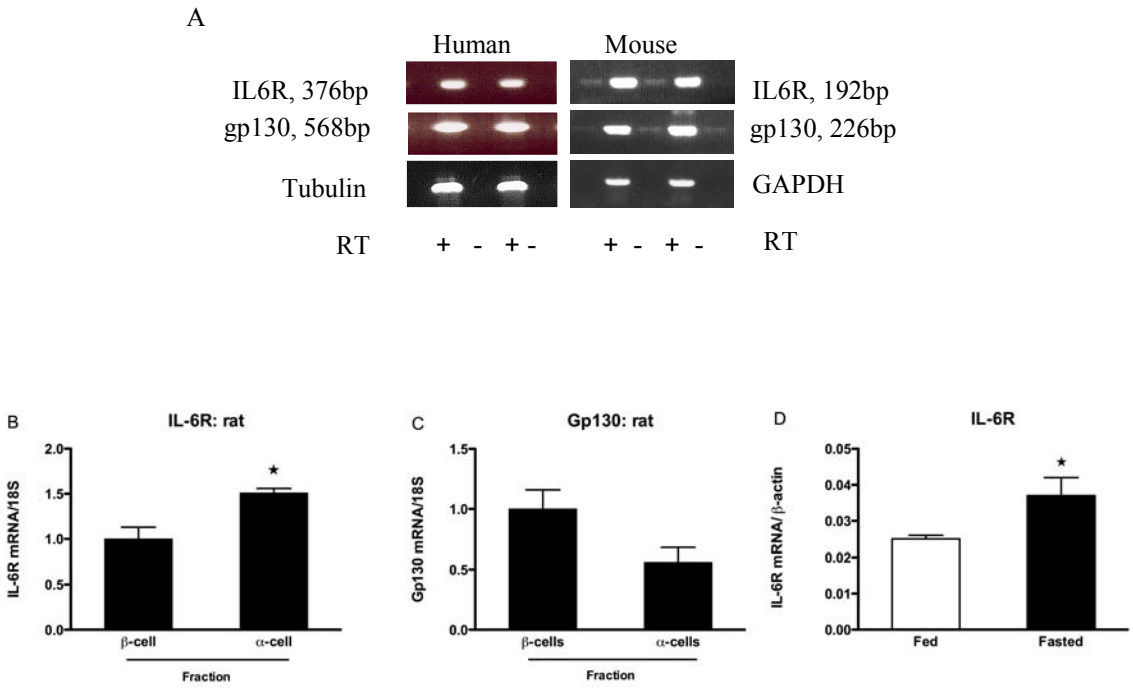
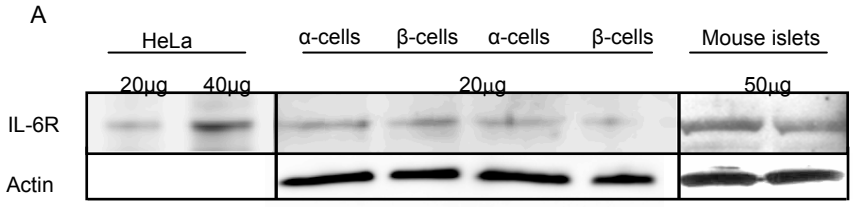


Figure 5: Interleukin-6 receptor (IL-6R) mRNA expression is enriched in the pancreatic α-cell. PCR on total RNA extracted from mouse and human islets using primers to detect the IL-6 receptor and gp130 expression in human and mouse islets, (+) and (-) represent cDNA and RT negative control respectively (A, representative of n=7). Quantitative RT-PCR on FACS sorted rat α-cells and β-cells using primers towards the IL-6 receptor (B) and gp130 (C). Purity about 90% as assessed by insulin and glucagon staining respectively. Results are normalized to 18S rRNA (n=3). Pancreatic islet IL-6 receptor mRNA expression in random fed and 12 hours fasted mice (D, n=4), results are normalized to β-actin. * $p<0.05$ by Student's t-test.



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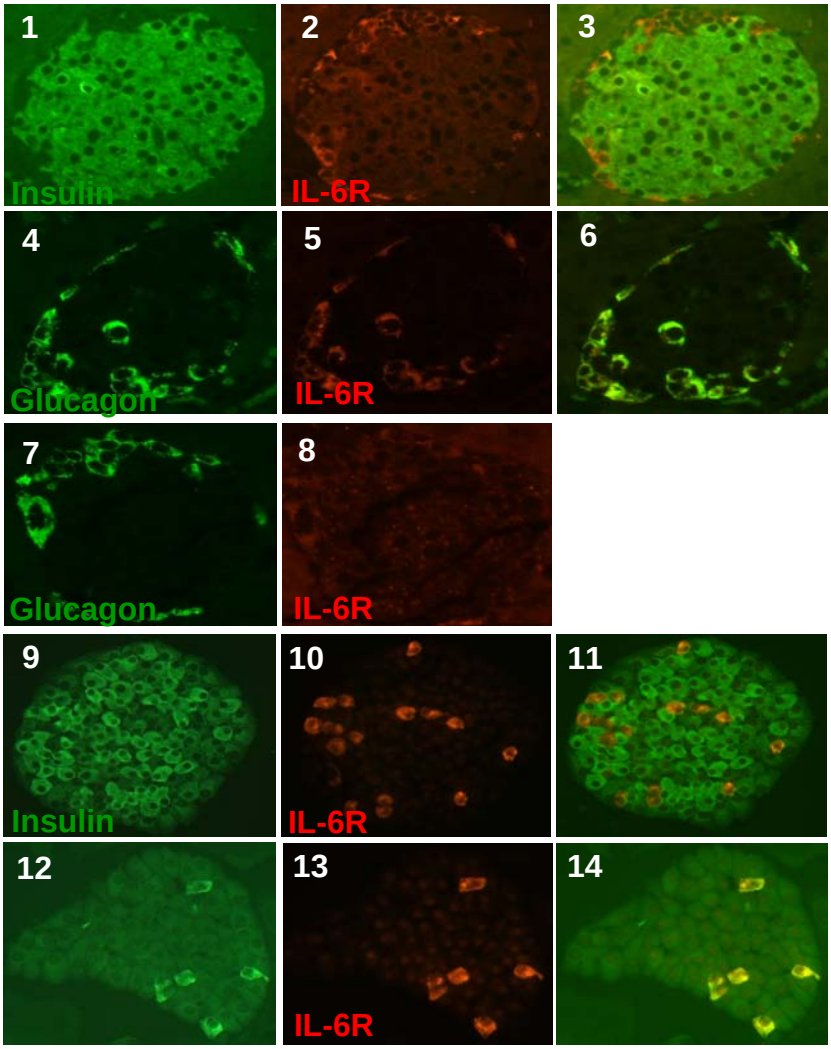

Figure 6: The Interleukin-6 receptor is localized to the pancreatic α-cell *in vivo*. Western blot analysis showing IL-6R in FACS sorted rat α-cells, β-cells, and mouse islets. Protein lysate from HeLa cells serves as a positive control, and actin serves as loading control (A, representative of n=3). Immunostaining of the IL-6R (panels 2, 5, 8, 10, 13) and the islet hormones insulin (panels 1, 9) and glucagon (panels 4, 7, 12) on mouse pancreatic tissue sections without (panels 1-6) and with pre-absorption using an IL-6R blocking peptide (panels 7, 8), and on cultured mouse islets (panels 9-14). (B, representative of n=5).

Functional expression of the IL-6 receptor was confirmed in mouse and human islets by acute (15 minutes) stimulation with 100 ng/ml IL-6. Western blot analysis revealed increased STAT3 phosphorylation, with no changes in the activation of pJAK2 and pERK (Figure 7).

Thus, based on gene array data, PCR**,** western blot analysis of FACS-sorted α-cells, and immunohistochemistry we localized the high level of IL-6 receptor expression in the pancreatic islet predominantly to the pancreatic α-cell *in vivo*, and found the IL-6 receptor to be functionally coupled to STAT3 phosphorylation. These data highlight the α-cell as a novel target of IL-6 actions.

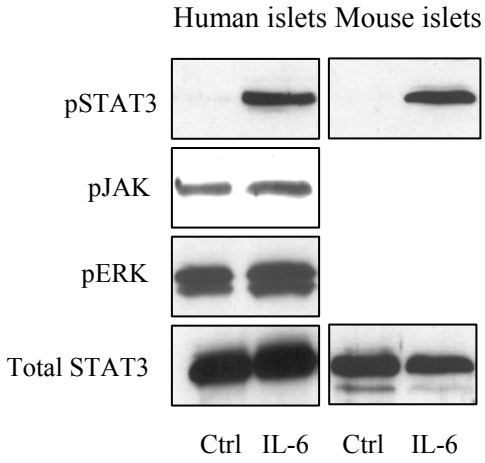

Figure 7: The Interleukin-6 receptor is functionally coupled to STAT3 phosphorylation in pancreatic islets. Western blot analysis of pSTAT3 (p=phosphorylated), pJAK2, pERK1/2, and total STAT3 in human and mouse islets after 15 min exposure to 100 ng/ml IL-6 (representative n=3).

CHAPTER 4: INTERLEUKIN-6 REGULATION OF GLUCAGON SECRETION AND ISLET CELL SURVIVAL *IN VITRO*

4.1 BACKGROUND

A role for IL-6 in regulating glucagon *in vitro* has never been reported, however, there exists evidence that IL-6 infusion in humans leads to an increase in plasma glucagon (Hiscock et al., 2005; Tsigos et al., 1997).

The mitogenic and growth promoting effects of IL-6 have never been investigated in pancreatic islet cells *in vitro*, however there are a wealth of studies on β-cells showing various growth promotive factors that signal through STAT3 (Garcia-Ocana et al., 2000; Nielsen et al., 2001; Yamamoto et al., 2000), substantiating the potential importance of this signalling pathway for the maintenance and expansion of islet mass. Pro-survival effects of IL-6 have been demonstrated in mouse islets and MIN-6 β-cells, and the authors report that cytokine induced apoptosis was prevented by IL-6 (Choi et al., 2004).

Thus, the following facts provided the rational for investigating whether IL-6 plays a role in the regulation of islet cell proliferation and/or apoptosis: 1) pancreatic islet cells express the IL-6 receptor, 2) a nutritional overload causes increased IL-6 release, 3) STAT3 is a key regulator of islet cell growth and survival, and finally 4) mitogenic and apoptotic effects of IL-6 on pancreatic islet cells is an undiscovered area.

4.2 RESULTS

4.2.1 Interleukin-6 regulation of proglucagon mRNA and glucagon secretion *in vitro*

Given the α-cell specific localization of the IL-6 receptor in pancreatic sections, initial experiments were targeted at elucidating whether IL-6 had an effect on glucagon production and secretion. Based on our own observations, pancreatic islets release IL-6 in the high pg/ml range (Figure 2BC); therefore IL-6 concentrations in the ng/ml range were used for all *in vitro* studies. This is supported by studies in hepatocarcinoma cells (Kim et al., 2008a) and neuronal cells (Krady et al., 2008) that also used IL-6 in the ng/ml range.

Human islets treated with 200 ng/ml IL-6 for 2, 4, 24, and 96 hours revealed a 2-fold increase in proglucagon mRNA after 4 hours (Figure 8A). The kinetic profile showed a peak in IL-6 induced proglucagon mRNA at 4 hours, followed by a gradual decline at 24 and 96 hours, displaying a tendency towards an IL-6 induced decrease in proglucagon mRNA after 96 hours (Figure 8A). The increase in proglucagon mRNA observed after 4 hours was followed by a significant more than 2-fold increase in glucagon release after 24 hours (Figure 8B). The specificity of these IL-6 effects were demonstrated by blocking IL-6 induced glucagon release from human islets with the IL-6 receptor

antagonist Sant7 (Sant7 is a molecular variant of IL-6 that binds specifically to the IL-6 receptor without initiating signaling). Control: 15.9 ± 3.6, IL-6: 36.9 ± 5.9, IL-6 + Sant7 : 15.8 ± 1.2 glucagon secretion as % of content (n=1 in quintuplicate)). To further investigate the specificity of the IL-6 effect on glucagon release, human islets were treated with 200 ng/ml of the IL-6 family member oncostatin M (OSM, which also uses gp130 as a signal transducer). These experiments supported a specific IL-6 effect, since OSM did not stimulate glucagon release from human islets (Control: 14.4 ± 1.5, OSM: 10.8 ± 4.4 glucagon secretion as % of content (n=2)). The time-dependent effect of IL-6 on glucagon release from human islets was also observed in mouse islets (Figure 8C). Twenty-four hour exposure to 1, 10, and 100 ng/ml IL-6 resulted in a significant increase in glucagon release at all doses. Notably, treatment of IL-6 knockout islets with 1 ng/ml IL-6 for 4 days caused a 5-fold increase in glucagon release. Importantly, no effect of IL-6 was observed on insulin mRNA and release in human islets at these time points (Chapther 5, figure 18AB).

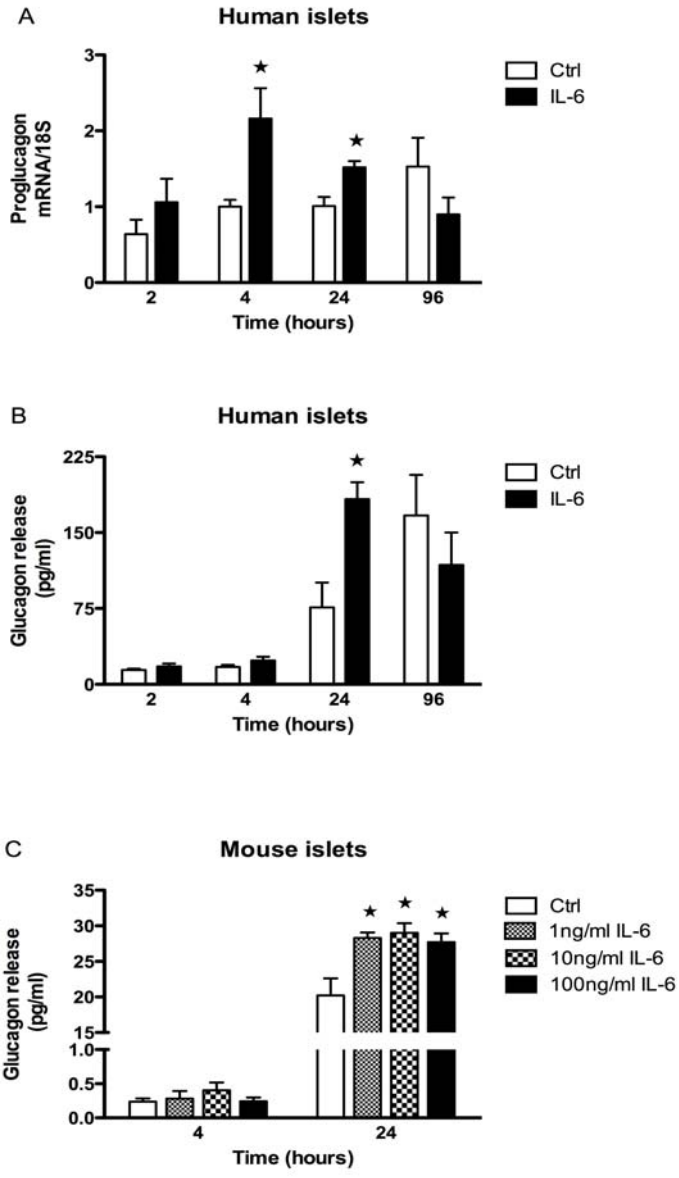

Figure 8: Interleukin-6 induces glucagon release from human and mouse pancreatic islets *in vitro*. Proglucagon mRNA (A, n=3) and glucagon release (100 and 20 islets/well respectively; B, n=7) in human islets cultured on ECM coated dishes for 2, 4, 24, and 96 hours in the absence (Ctrl, white bars) and presence of 200 ng/ml IL-6 (black bars). Glucagon release from mouse islets (30 islets/well; C, n=3) cultured on ECM coated dishes for 4 and 24 hours in the absence (Ctrl, white bars) and presence of IL-6. * $p<0.05$ by Student's t-test (A and B versus Ctrl), and ANOVA with Dunnett's post-hoc test (C, versus Ctrl).

Along with the novel observation that IL-6 regulated proglucagon mRNA and glucagon release, we considered the possibility that IL-6 might regulate α-cell glucagon secretion in response to low versus high glucose. Therefore, subsequent experiments were aimed at elucidating whether IL-6 affected acute glucagon secretion induced by (low) glucose and arginine, and thus α-cell secretory function. To address this question human islets were pre-incubated for 4 and 24 hours in the absence or presence of 200 ng/ml IL-6, followed by a 1 hour static incubation in either high glucose (20 mM), representing an unstimulated condition leading to basal glucagon secretion, or low glucose (2 mM), a condition stimulating glucagon secretion in whole islets (Gromada 2007), or in the presence of 10 nM arginine known to induce a rapid increase in glucagon release. Arginine induced glucagon release is thought to be a consequence of the ability of arginine to depolarize the α-cell (due to its positive charge) and cause an unspecific release of glucagon (Gromada J 2004 Diabetes; Göpel SO 2000 J Physiol). IL-6 preincubation for both 4 and 24 hours caused increased glucagon secretion under both unstimulated (20 mM) and stimulated (2 mM) glucose conditions, however, there was no significant effect on arginine-stimulated glucagon secretion (Figure 9A). This is indicative of an IL-6-induced improved responsiveness of the pancreatic α-cell to low versus high glucose conditions with respect to glucagon secretion, with no effect on total glucagon content. In support of this, we did not see an effect of IL-6 on islet glucagon content (Figure 9B).

In order to support these *in vitro* findings *in vivo*, a bolus injection of IL-6 (100 ng) was administered into conscious mice and circulating glucagon levels were monitored over time. Initially, a time course was conducted to look at IL-6 induction of glucagon after 2, 4, and 6 hours (not shown). Injection of IL-6 increased glucagon levels after 2 hours only during fasting conditions, with no effect during the fed state (Figure 9C).

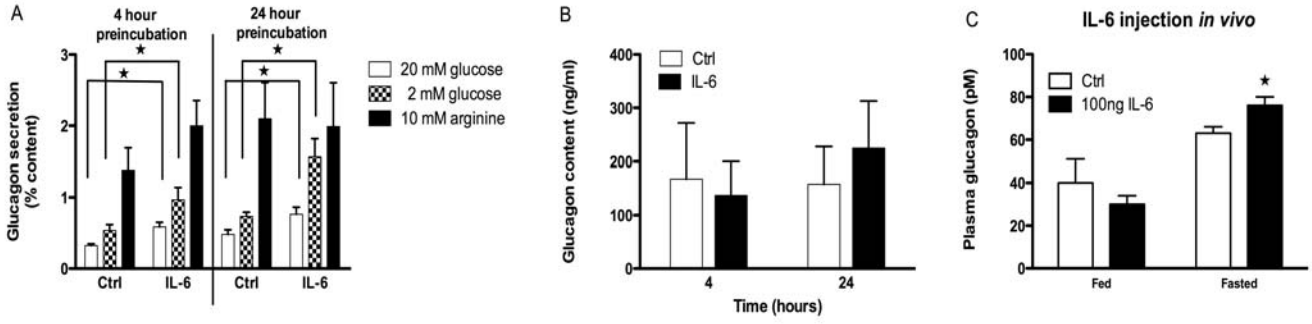

Figure 9: Interleukin-6 regulates pancreatic α-cell glucagon secretion in response to glucose. (A) Glucagon secretion from human islets during a 1 hour static incubation on ECM coated dishes with 20 mM glucose, 2 mM glucose, and 10 mM arginine. Islets were pretreated for 4 and 24 hours in the absence (Ctrl) or presence of 200 ng/ml IL-6 (n=4). (B) Glucagon content in human islets after 4 and 24 hours treatment on ECM coated dishes in the absence, or presence of 200 ng/ml IL-6 (n=4). (C), Plasma glucagon in random fed and 12 hour fasted mice after a bolus intraperitoneal injection with saline (Ctrl) and 100 ng IL-6 (n=6). * $p<0.05$ versus respective controls, as tested by Student's t-test.

Thus, the α-cell is a target of IL-6 actions *in vitro*. Interelukin-6 pre-treatment of islets improves their ability to secrete glucagon in response to low glucose, an effect also found *in vivo*.

4.2.2 Interleukin-6 regulation of islet cell proliferation *in vitro*

Experiments aimed at elucidating whether IL-6 had an effect on islet cell proliferation were initially conducted on intact mouse and human islets, without identification of the proliferating islet cell type. Proliferation was assessed with Ki67 immunostaining, however the necessity to recognize the complete proliferative activity throughout the treatment period led to the use of BrdU incorporation.

Human islets cultured for 4 days in the presence of 200 ng/ml IL-6 showed a remarkably consistent 4-fold increase in proliferating islet cells per islet, both when assessed with Ki67 staining and with BrdU incorporation (Figure 10 AB). The continuous labeling of proliferating cells with BrdU resulted in absolute numbers that were 4-fold higher than when assessed with Ki67. Given the fact that human islets are capable of producing and releasing IL-6, we hypothesized that endogenous IL-6, acting in an autocrine and/or paracrine manner could induce islet cell proliferation. Therefore, human islets were cultured in the presence of an IL-6 receptor antagonist (Sant7) revealing a decrease in proliferation with increasing doses of Sant7 (Figure 10C). Thus, both exogenous and endogenous IL-6 is capable of inducing islet cell proliferation in human islets. Based on the observed stimulatory effect of IL-6 on glucagon secretion, it was hypothesized that in addition to IL-6, increased glucagon levels could induce islet cell proliferation *in vitro*. The speculation was that IL-6 induced a direct proliferative signal through the IL-6 receptor, followed by an indirect proliferative signal by glucagon acting through the glucagon receptor on β-cells to cause β-cell proliferation. Indeed, these experiments revealed that exogenous glucagon (10 nM) is capable of inducing islet cell proliferation, however with a tendency for glucagon to be less potent than IL-6 (Figure 10D). Thus, IL-6 presents itself as a strong inducer of islet cell proliferation in human islets *in vitro*.

The importance of the above described results is partly related to the fact that they are conducted on human islets. However, along with initiating studies on mutant mice, the necessity of characterizing IL-6 effects on proliferation in mouse islets was obvious. Mouse islets treated with 1-100 ng/ml IL-6 in the presence of BrdU for 4 days showed an increase in islet cell proliferation in a dose-dependent manner, with the most profound effect being a 3-fold increase (Figure 11A). A time-course evaluating the effect of IL-6 on islet cell proliferation in intact islets after 2, 3, and 4 days revealed that only after 4 days treatment, a significant increase in proliferation was observed, with a tendency towards an increase after 3 days (Figure 11B). Furthermore, IL-6 induced islet cell proliferation in isolated islets from IL-6 knockout mice, demonstrated that IL-6 is still able to cause proliferation when basal IL-6 release from islets is removed (Figure 11C). Interestingly, basal mouse islet cell proliferation was significantly higher compared to basal human islet cell proliferation, and further IL-6 induced proliferation was slightly more pronounced in human islets.

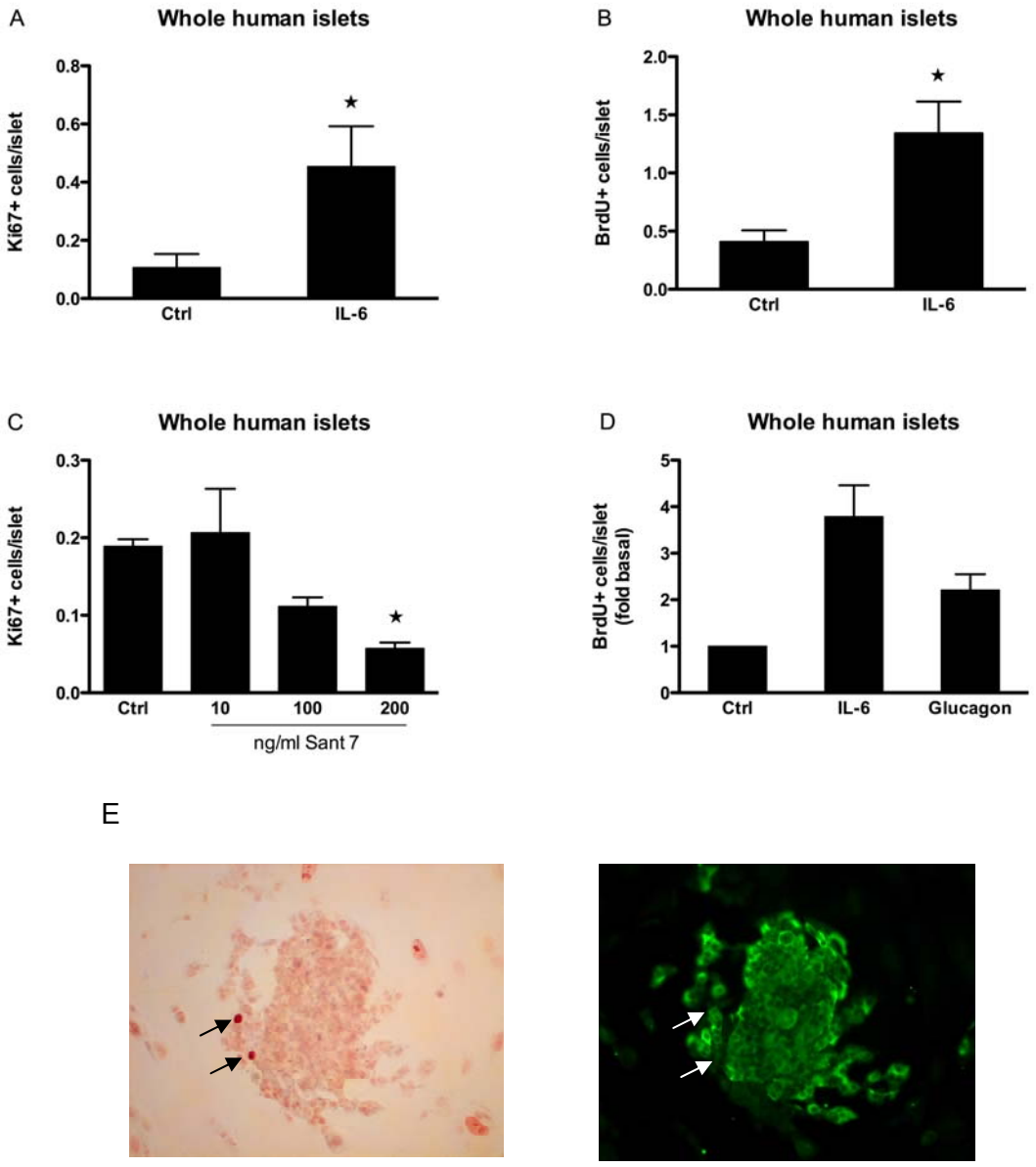

Figure 10: Interleukin-6 induces proliferation of human islet cells. Ki67 (A,C) and BrdU (B,D) positive human islet cells per islet after 4 days treatment on ECM coated dishes in the absence (Ctrl) and presence of 200 ng/ml IL-6 (A,B, n=3-5), and in the presence of 1, 10, 100 ng/ml of the IL-6R antagonist Sant7 (C, n=3), and 10 nM exogenous glucagon (D, n=2). A final concentration of 10 μM BrdU was present during the entire treatment period. (E) A representative human islet stained for Ki67 (red nuclei, left panel), and insulin (green cytoplasm, right panel). * $p<0.05$ by Student's t-test (A, B), and ANOVA with Dunnett's post-hoc test (C, versus Ctrl).

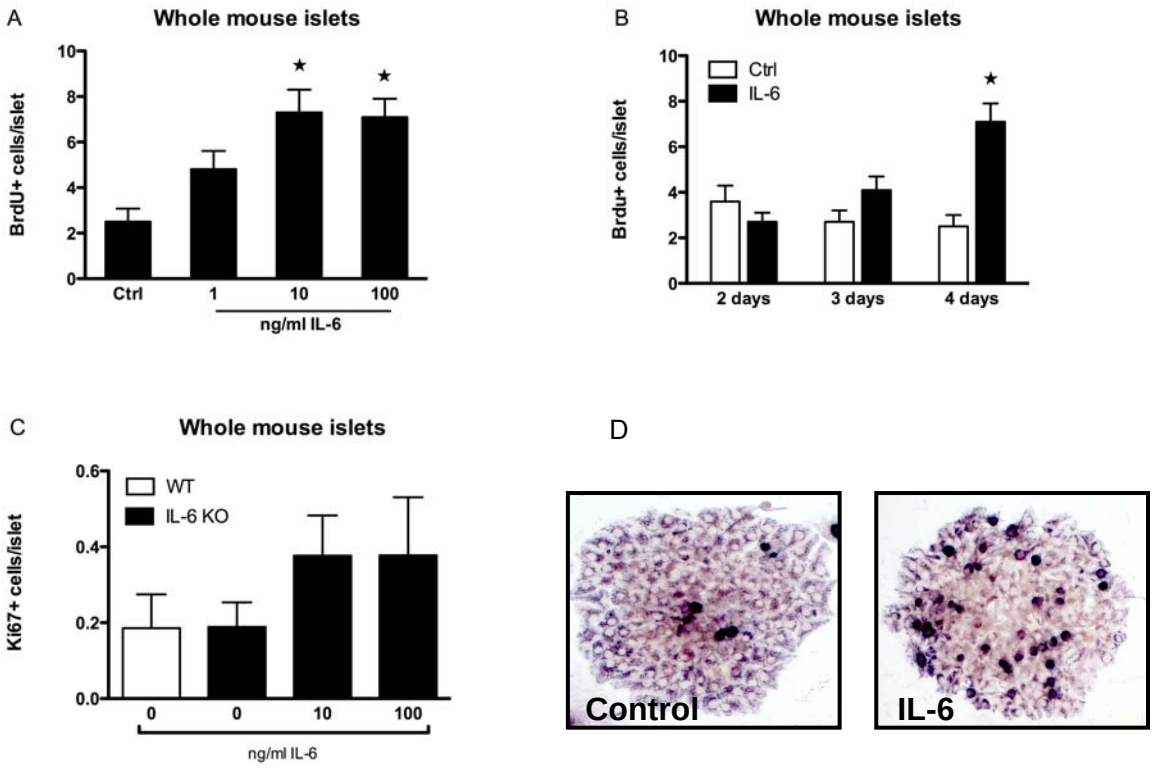

Figure 11: Interleukin-6 induces proliferation of mouse islet cells. (A) BrdU positive cells per islet in mouse islets treated on ECM coated dishes with 1, 10, 100 ng/ml IL-6 for 4 days (n=3). (B) BrdU positive cells per islet in mouse islets treated on ECM coated dishes with IL-6 (100 ng/ml) for 2, 3, and 4 days. (C) Ki67 positive islet cells in wild type (WT) and IL-6 knockout (IL-6 KO) islets after 4 days treatment in the absence (0) or presence (10 or 100 ng/ml IL-6) (n=1, performed in triplicate). (D) Images of mouse islets treated on ECM coated dishes in the absence (Control) or presence of 100 ng/ml IL-6. A final concentration of 10 μM BrdU was present during the entire treatment period. * $p<0.01$ by Student's t-test (B), and *$p<0.05$ as tested by ANOVA with Dunnett's post hoc-test (A, versus Ctrl).

4.2.3 Intereukin-6 regulation of α-cell and β-cell proliferation *in vitro*

The experiments conducted so far provide evidence for IL-6 as a strong inducer of islet cell proliferation. The subsequent aim was targeted at identifying the identity of the proliferating cells. 90% pure fractions of FACS sorted rat α-cells and β-cells treated for 48 hours, revealed a significant increase in both α-cell and β-cell proliferation (Figure 12A). The ensuing experiment was therefore conducted to investigate the kinetic profile of IL-6 induced proliferation in different endocrine cell types. Intact mouse islets were treated for 24 and 96 hours, and dispersed into single cells at the end of the experiment. These experiments showed that already after 24 hours 100 ng/ml IL-6 caused a 3-fold increase in α-cell proliferation, with no effect on β-cell proliferation at this time point (Figure 12BC). At 96 hours, the increase in α-cell proliferation observed at the earlier time point was masked by an increased basal proliferation, however at this time point IL-6 caused a significant greater than 3-fold increase in β-cell proliferation (Figure 12BC). Thus, IL-6 presents itself as a strong regulator of endocrine cell proliferation in human, mouse and rat islets, with the earliest effects occurring on α-cells.

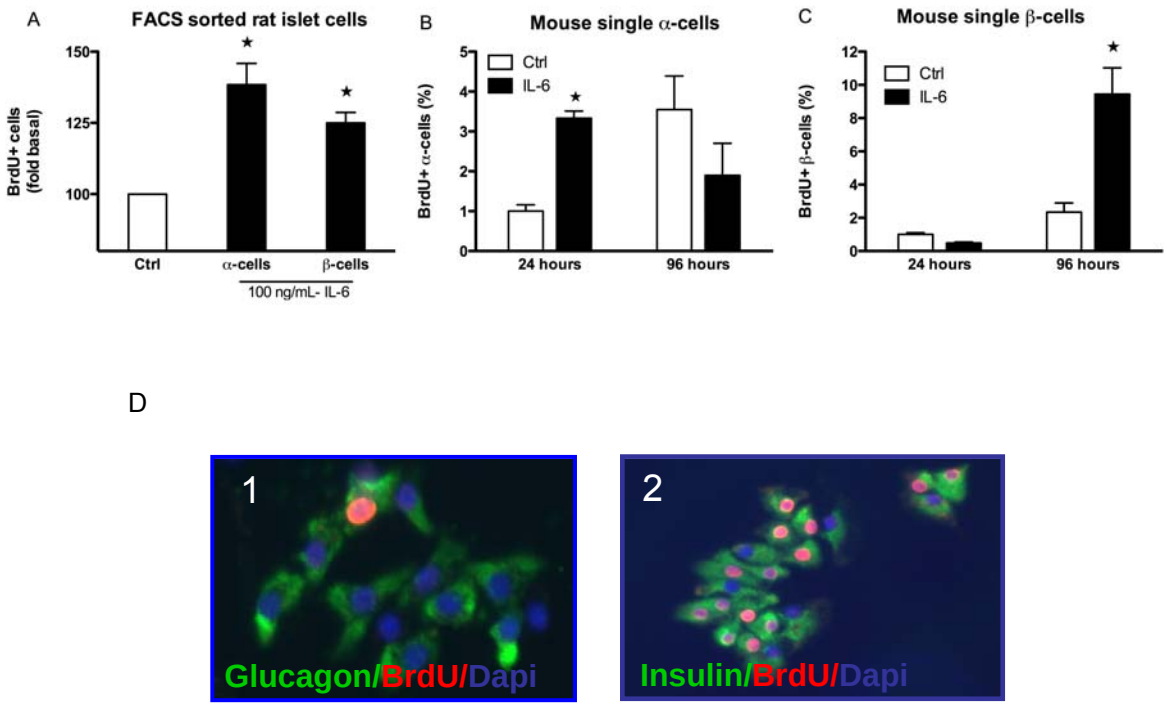

Figure 12: Interleukin-6 induces pancreatic α-cell and β-cell proliferation *in vitro*. (A) BrdU positive FACS sorted rat α-cells and β-cells treated on 804G-ECM dishes in the absence (Ctrl) and presence of 100 ng/ml IL-6 for 48 hours; results are expressed as fold basal (n=3). A final concentration of 10 μM BrdU was present during the entire treatment period. Percentage BrdU positive mouse α-cells (B), and β-cells (C) treated on ECM coated dishes in the absence (Ctrl) and presence (IL-6) of 100 ng/ml IL-6 for 24 and 96 hours (n=3). In panels B and C islets were dispersed at the end of the experiment. (D) Images of FACS sorted rat α-cells (panel 1) and β-cells (panel 2) stained for dapi (blue), Brdu (red), and insulin (green). * $p<0.05$ as tested by ANOVA with Dunnett's post-hoc (A, versus Ctrl), and by Student's t-test (C).

4.2.4 Interleukin-6 protection against nutrient induced α-cell apoptosis *in vitro*

The first experiments aimed at uncovering a role for IL-6 in islet cell apoptosis were conducted on intact human and mouse islets. Human islets treated for 4 days with 200 ng/ml IL-6 showed a tendency towards an increase in apoptotic islet cells (Figure 13A). In one single experiment (done in triplicate) with successful double staining for BrdU and either glucagon or insulin, we observed a distinct effect on α-cells and β-cells. In α-cells, IL-6 decreased basal level of apoptosis (Figure 13B), whereas in β-cells IL-6 induced apoptosis (Figure 13C). In mouse islets there was no effect on apoptosis in intact islets treated with 1 - 100 ng/ml IL-6 for 4 days (Figure 14A), however in mouse single cells 100 ng/ml IL-6 caused a 12-fold increase in apoptotic β-cells after 96 hours treatment, while there was no effect of IL-6 on α-cell apoptosis in these same experiments (Figure 14BC). Interestingly, basal level of apoptosis was higher in α-cells compared to β-cells. Comparing the level of apoptosis in wild type and IL-6 knockout islets after 4 days exposure to 5.5 mM, 11.1 mM and 33.3 mM glucose revealed a reduced number of apoptotic islet cells in the IL-6 knockout islets at

all glucose concentrations. Furthermore, in both wild type and IL-6 knockout islets the typical u-shaped curve revealing increased number of apoptotic islet cells at 5.5 mM and 33.3 mM as compared to 11.1 mM glucose was demonstrated (Figure 14D).

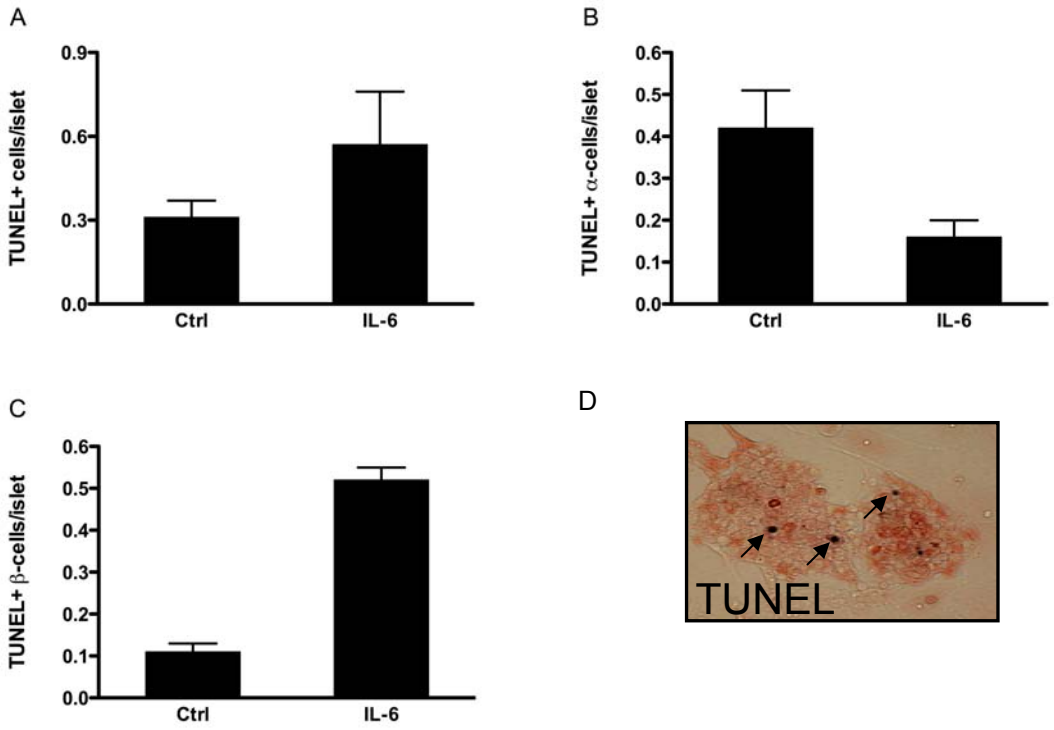

Figure 13: Interleukin-6 regulation of islet cell apoptosis in whole human islets. TUNEL positive islet cells (A, n=6), α-cells (B, n=1 in triplicate), and β-cells (C, n=1 in triplicate) in human islets treated on ECM coated dishes in the absence (Ctrl) and presence of 200 ng/ml IL-6 for 4 days. Islets were stained for TUNEL only (A), and co-stained with glucagon (B), or insulin (C). (D) Image of a human islet stained for TUNEL (black nuclei, black arrows) and insulin (red cytoplasm).

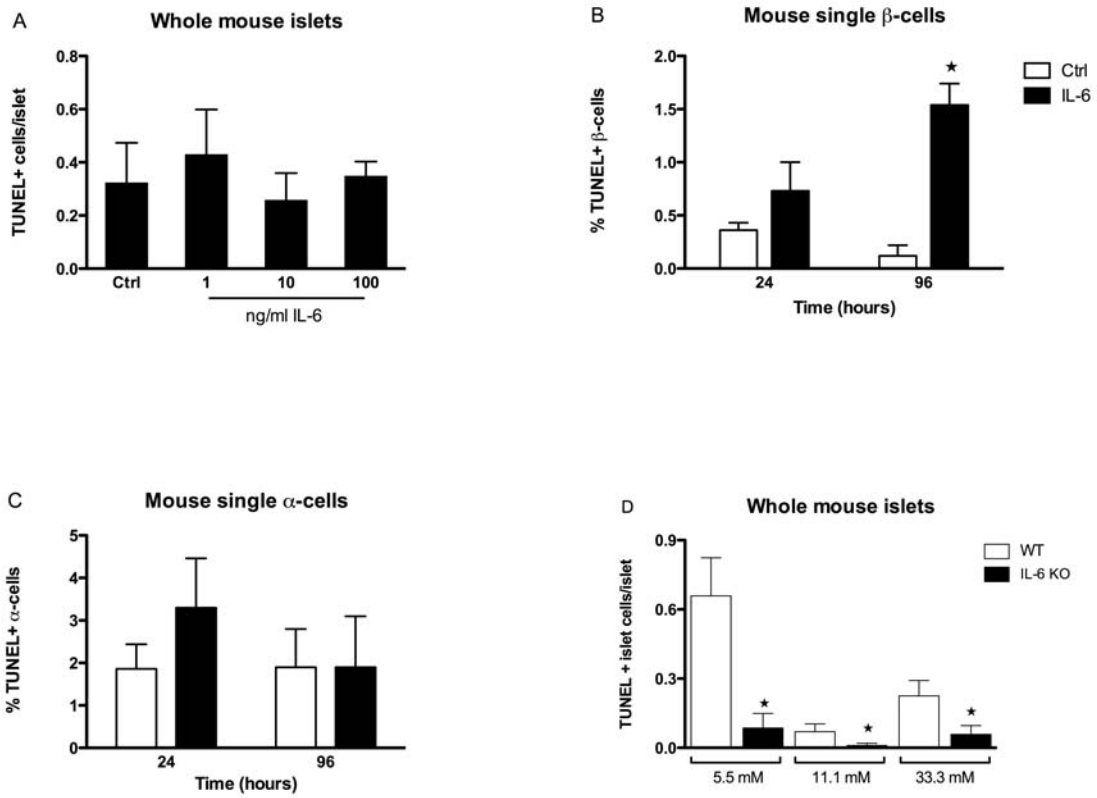

Figure 14: Interleukin-6 has no effect on islet α-cell apoptosis, but increases β-cell apoptosis. (A) TUNEL positive mouse islet cells treated on ECM coated dishes in the absence (Ctrl) or presence of increasing doses of IL-6 for 4 days (n=5). (B,C) % TUNEL positive mouse β-cells (B, n=3) and α-cells (A, n=3) treated on ECM coated dishes as single cells for 24 and 96 hours in the absence (Ctrl) or presence of 100 ng/ml IL-6 (IL-6). (D) TUNEL positive islet cells in islets from wild type (WT) and IL-6 knockout islets (IL-6 KO) after 4 days in the presence of 5.5, 11.1, or 33.3 mM glucose (n=3). *$p<0.05$ by Student's t-test versus respective Ctrl or wild type.

In previous studies IL-6 had been shown to have an anti-apoptotic effect on MIN-6 β-cells and mouse islet cells (Choi et al., 2004). To investigate this possibility in a context relevant for type 2 diabetes, apoptosis was induced in dispersed mouse single cells by exposure to a nutritional overload (0.5 mM palmitate (16:0) and high 33.3 mM glucose) for 12 hours, with and without pre-incubation with IL-6. The combination of palmitate and glucose induced a significant increase in α-cell apoptosis, an effect that was prevented by pre-incubation with IL-6 (Figure 15A). The effect of palmitate plus glucose on β-cell apoptosis was rather mild (possibly due to short exposure time), and interestingly, the presence of IL-6 induced a significant increase in number of apoptotic β-cells (Figure 15B). Thus, these results reveal a distinct role of IL-6 in regulating apoptosis in pancreatic α-cells and β-cells.

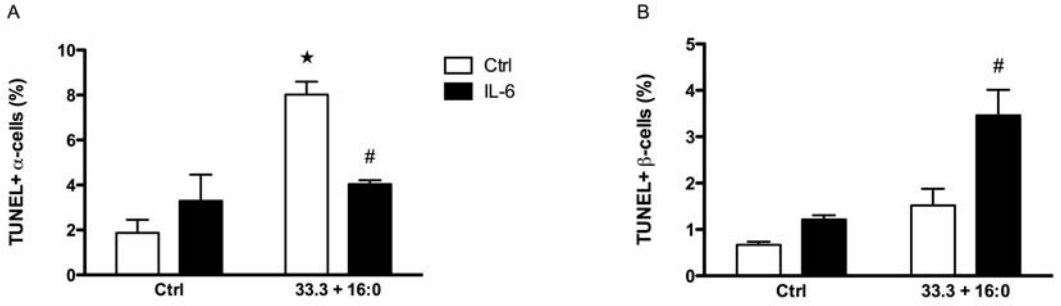

Figure 15: Interleukin-6 has distinct effects on nutrient induced apoptosis in α-cells and β-cells. (A) Percentage TUNEL positive α-cells (A, n=3) and β-cells (B, n=3) in mouse islet cells dispersed into single cells prior to treatment on ECM coated dishes for 12 hours with 0.5 mM palmitate (16:0) and 33.3 mM glucose (33.3+16:0). Cells were pretreated with 100 ng/ml IL-6 (IL-6) for 1 hour prior to adding conditions. At the end of the experiment cells were double stained for TUNEL and glucagon (A) or insulin (B). * $p<0.05$ by Student's t-test and by ANOVA with Dunnett's post hoc-test (A, versus Ctrl).

4.2.5 Interleukin-6 induces proliferative and pro-survival genes *in vitro*

Finally, we investigated the mechanisms possibly involved in the proliferative and anti-apoptotic effects of IL-6 on the α-cell. Quantitative RT-PCR revealed an IL-6 induced increase in c-myc, and Bcl-2 expression, and a decrease in the cell cycle inhibitor p27, however no effects were observed on mRNA levels of the D cyclins 1,2, and 3 (Figure 16A-F).

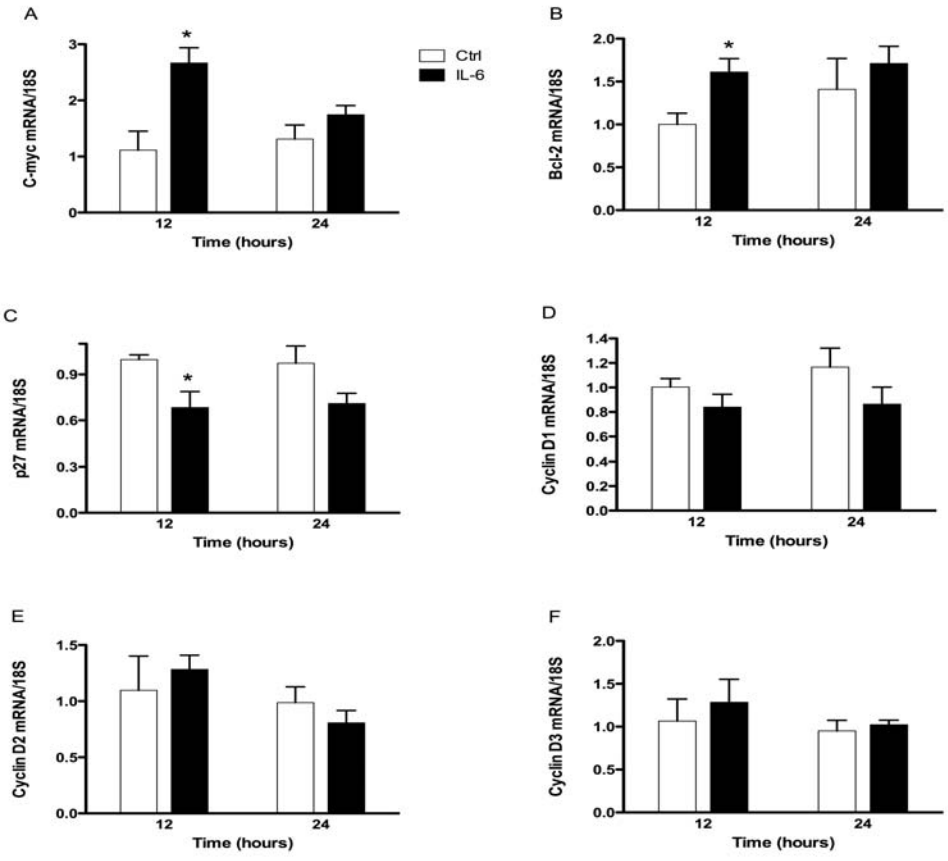

Figure 16: Short-term IL-6 treatment induces proliferative and prosurvival genes in mouse islets. mRNA expression of c-myc (A), Bcl-2 (B), p27 (C), cyclin D1(D), cyclin D2 (E), cyclin D3 (F) in mouse islets treated for 12 and 24 hours on ECM coated dishes in the absence (Ctrl) and presence (black bars) of 100 ng/ml IL-6 (n=3).
* $p<0.05$ by Student's t-test.

CHAPTER 5: INTERLEUKIN-6 REGULATION OF PANCREATIC β-CELL FUNCTION *IN VITRO*

5.1 BACKGROUND

Previous work investigating the effects of IL-6 on pancreatic islet insulin secretion and β-cell function has provided confounding results. However, the majority of studies conducted on rat islets report a detrimental effect of IL-6 on glucose stimulated insulin secretion (Sandler et al., 1990; Southern et al., 1990; Wadt et al., 1998), and a recent paper conducted on mouse islets also revealed impaired glucose stimulated insulin secretion in mouse islets (Handschin et al., 2007), whereas no effect of IL-6 on β-cell function has been reported in human islets (Choi et al., 2004; Eizirik et al., 1994). RIP-IL-6 mice display normal glucose tolerance, and as our results indicate, lack of IL-6 does not affect β-cell function under normal conditions. In addition, infusion of IL-6 in healthy subjects has no significant effect on plasma insulin levels (Tsigos et al., 1997; van Hall et al., 2003). Thus, we aimed to clarify the effects of IL-6 on pancreatic β-cell function *in vitro*.

5.2 RESULTS

Based on the above studies, IL-6 was found to regulate α-cell function very early kinetically during treatment. Therefore, these studies were paralleled by looking at both IL-6 regulation of insulin mRNA and secretion directly, and whether IL-6 regulates glucose-stimulated insulin secretion at various times of treatment.

Initial experiments were aimed at elucidating whether IL-6 regulated insulin secretion directly. Both mouse and human islet data revealed no insulinotropic effects of IL-6 during a 1 hour static incubation in the presence of 7.5 mM glucose levels in addition to increasing doses of IL-6 (Figure 17AB). Further, IL-6 incubation of human islets from 4 hours to 4 days showed no effects on insulin mRNA or insulin release (Figure 18AB).

Previous studies performed in rat islets showed an inhibitory effect of IL-6 on β-cell glucose stimulated insulin secretion, however these effects were both dose- and time-dependent (Sandler et al., 1990; Southern et al., 1990; Wadt et al., 1998). Therefore, a variety of doses and time-points were chosen for the present experiments. Furthermore, in some experiments IL-6 knockout islets were used to avoid the complication of endogenous IL-6 release. Interleukin-6 knockout islets were isolated from animals at 10 - 12 weeks of age, where no dysfunctional islet phenotype was reported (Chapter 6, figure 22C).

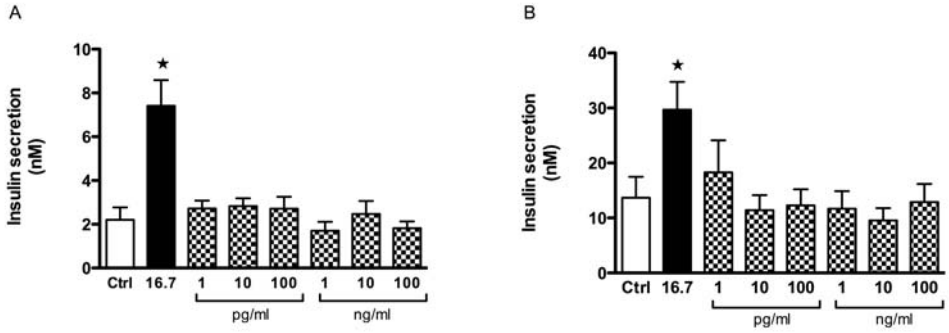

Figure 17: Interleukin-6 does not stimulate insulin secretion acutely *in vitro*. Insulin secretion in mouse (A, n=3) and human (B, n=3) islets (20 islets/well) during a 1 hour static incubation in KRBB containing 7.5 mM glucose (Ctrl), 16.7mM glucose (black bars) or 7.5 mM glucose with increasing doses of IL-6. *p<0.05 by ANOVA with Dunnett's post-hoc test versus Ctrl.

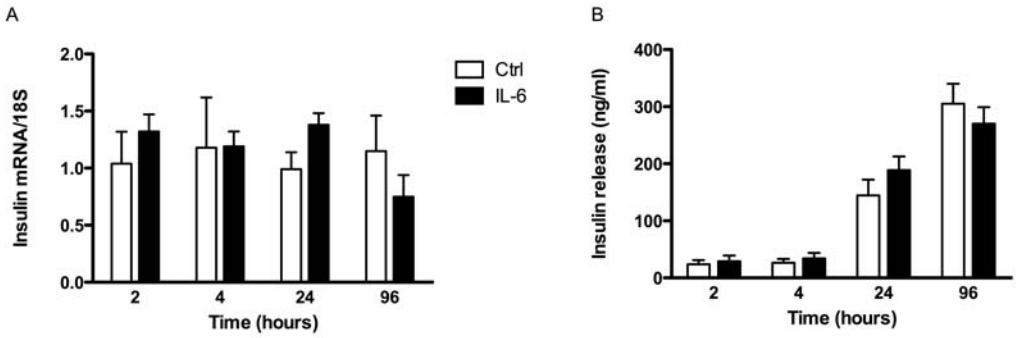

Figure 18: Interleukin-6 has no effect on insulin mRNA or release from human islets. Insulin mRNA (A, n=3-7), and insulin release (B, 20 islets/well; n=3-7) in human islets after 2, 4, 24, and 96 hours treatment in the absence (Ctrl) and presence of 200 ng/ml IL-6 (IL-6).

Although insulin secretion was not directly affected by IL-6 treatment, it was investigated whether glucose-stimulated insulin secretion may be regulated (in a similar manner to glucose regulated glucagon secretion was regulated above). Choosing a dose similar to that used on human islets, a time-course conducted in mouse islets revealed that only after 3 days was there a significant impairment in β-cell function in response to 100 ng/ml IL-6 (Figure 19EF). Therefore, mouse islets from IL-6 knockout mice were treated with increasing doses of IL-6 for 4 days to assess the dose-dependence of IL-6 effects on insulin secretion in the absence of confounding IL-6 release from islets (Figure 19AB). At the same time, effects on wild type mouse islets were confirmed. Treated islets showed an impaired ability to secrete insulin in response to 4 days treatment with IL-6. These effects were dose-dependent, and primarily due to a decrease in glucose stimulated insulin secretion rather than an increased basal insulin release (Figure 19AB). Further, these effects were supported by data on mouse MIN-6 β-cells, where the inhibitory effect of 4 days IL-6 treatment on insulin secretion was due to both an increased basal insulin release, and a decreased in glucose stimulated insulin secretion (Figure 19CD). Thus, chronic elevated IL-6 impairs glucose stimulated insulin secretion from mouse islets.

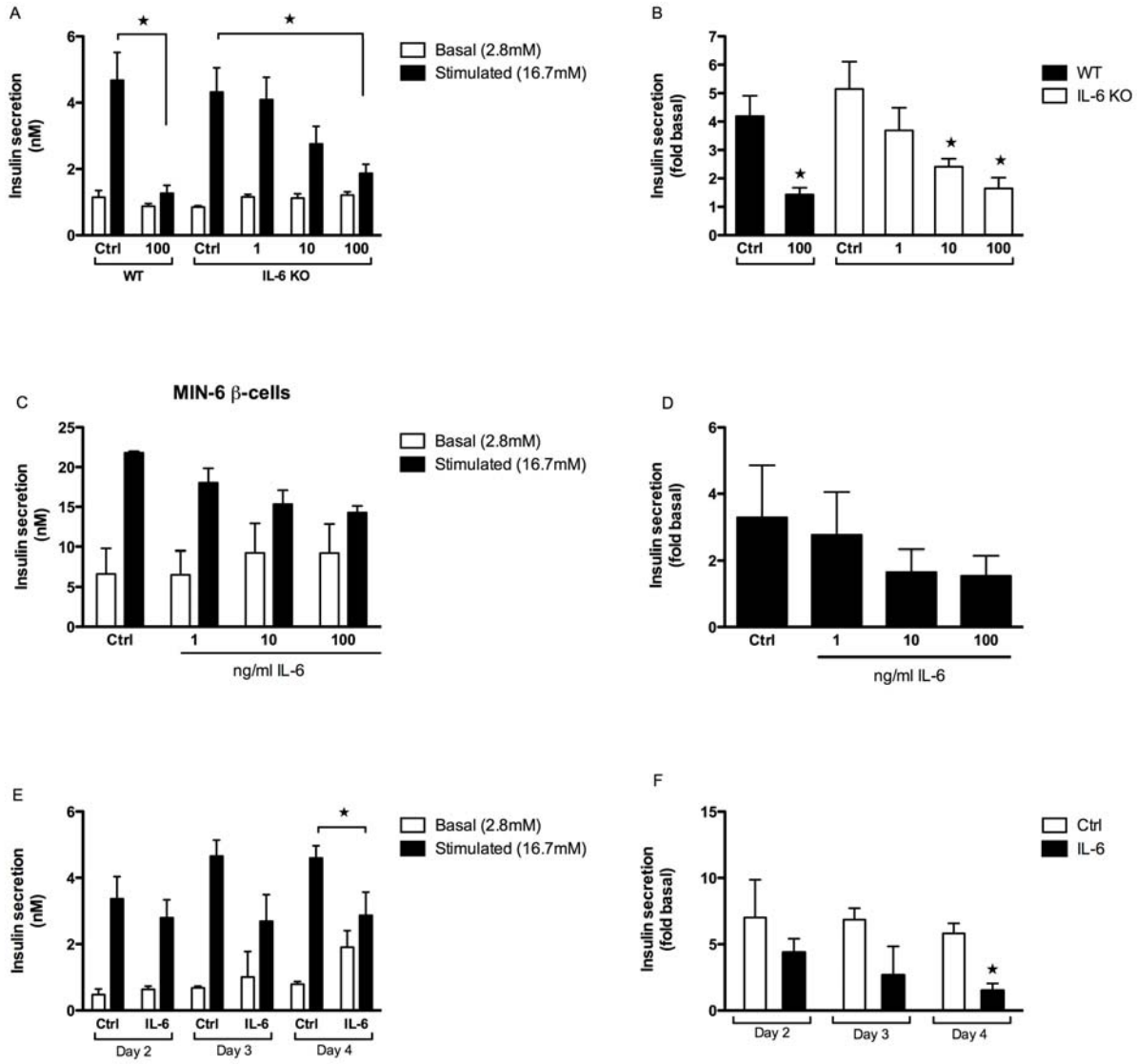

Figure 19: Interleukin-6 impairs β-cell function in mouse islets *in vitro*. Glucose stimulated insulin secretion (GSIS) during 1 hour static incubation in KRBB containing 2.8 mM glucose (Basal) and 16.7 mM glucose (Stimulated) after incubation of mouse islets (20 islets/well) in the absence (Ctrl) or presence of increasing doses of IL-6 for 4 days (ng/ml) (A: WT and IL-6 KO, n=4). The fold increase (stimulated insulin secretion/basal) is shown in (B). GSIS in MIN-6 β-cells (C, n=3) after 4 days treatment in the absence (Ctrl) or presence of increasing doses of IL-6. Fold increase is shown in (D). GSIS in mouse islets after 2, 3, and 4 day incubation with 100 ng/ml IL-6 (E, n=3); fold increase is shown in (F). *$p<0.05$ by Student's t-test versus untreated 16.7 mM glucose stimulated Ctrl.

In the literature there are reported species differences with respect to IL-6 effects on insulin secretion, therefore it was important to perform the above experiments on human islets. Similar to mouse islets, 4 day treatment with 200 ng/ml IL-6 cause a decrease in glucose stimulated insulin secretion in human islets, with no effect on basal insulin secretion (Figure 20A). This observed effect was not due to IL-6 induced changes in insulin content, although there was a tendency towards a decrease due to IL-6 treatment (Figure 20C). To clarify the specificity of the detrimental effects of IL-6, experiments using the IL-6 receptor antagonist Sant7 were performed. Islets were pre-treated for 2 hours prior to adding exogenous IL-6, and these experiments proved that the observed inhibitory

effects of IL-6 on glucose stimulated insulin secretion in human islets were specific to IL-6 (Figure 21A). In view of the fact that islets are capable of releasing IL-6 it was hypothesized that endogenous IL-6 could act in an autocrine/paracrine manner to impair β-cell function. To address this question, human islets were treated in the presence of Sant7 only, and these results revealed that endogenous IL-6 is detrimental to β-cell function *in vitro* (Figure 21B).

Human islet preparations, in contrast to mouse islets, release vastly variable amounts of IL-6, and furthermore also present with extremely variable insulin secretion in response to glucose. Based on the above experiments, it was hypothesized that there might be a correlation between basal IL-6 release and β-cell function. In agreement with the above data, a negative correlation between basal islet derived IL-6 and β-cell insulin secretion in response to elevated glucose exists in human islets (Figure 20D).

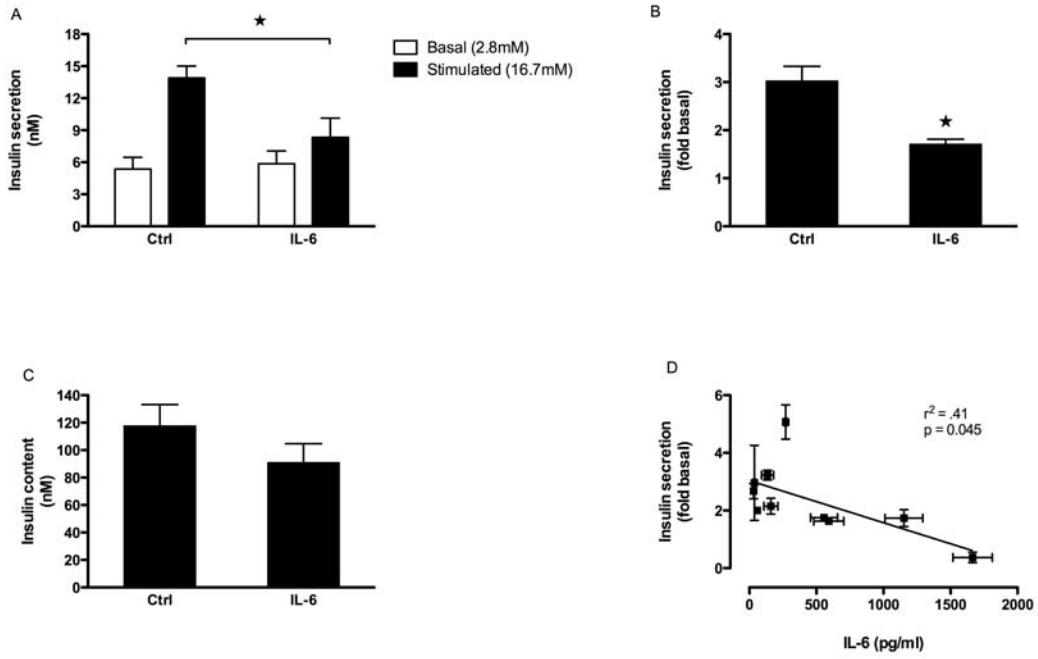

Figure 20: Interleukin-6 impairs β-cell function in human islets *in vitro*. (A) Glucose stimulated insulin secretion (GSIS) during 1 hour static incubation in KRBB containing 2.8 mM glucose (Basal) and 16.7 mM glucose (Stimulated) in human islets after 4 days treatment on ECM coated dishes in the absence (Ctrl) and presence of 200 ng/ml IL-6. (B) Results expressed as fold increase (stimulated insulin secretion/basal insulin secretion). (C) Insulin content (extracted with 0.18 M HCl in 70% ethanol) in human islets treated for 4 days on ECM coated dishes in the absence (Ctrl) and presence of 200 ng/ml IL-6. (D) Correlation between basal IL-6 release from human islets (48 hours culture) and β-cell function assessed by GSIS (n=10). *p<0.05 by Student's t-test versus untreated 16.7 mM glucose stimulated control.. Linear regression was performed (D) using the data analysis program Graphpad Prism.

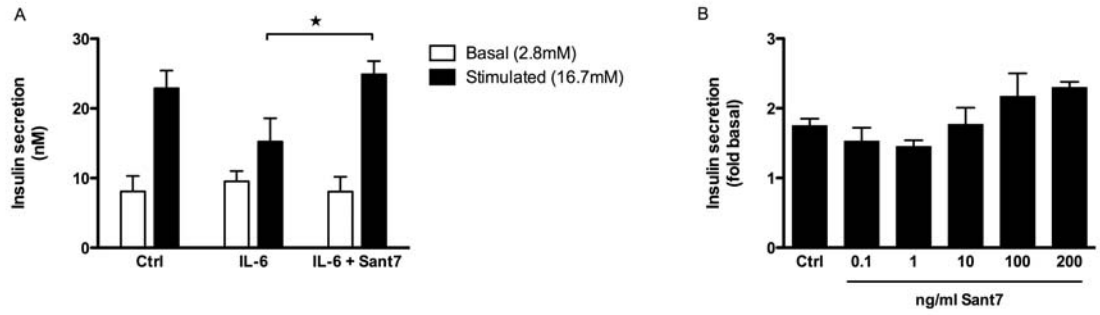

Figure 21: Endogenous islet derived IL-6 release impairs β-cell function in human islets *in vitro*. Glucose stimulated insulin secretion (GSIS) during 1 hour static incubation in KRBB containing 2.8 mM glucose (Basal) and 16.7 mM glucose (Stimulated) in human islets (20 islets/well) after 4 days treatment on ECM coated dishes in the absence (Ctrl) and presence of 200 ng/ml IL-6 and 200 ng/ml Sant7 (2 hours pretreatment) (A, n=3). Glucose stimulated insulin secretion in human islets treated for 4 days in the absence (Ctrl) or presence of 1-200 ng/ml of the IL-6 receptor antagonist Sant7 (B, n=4). Results are expressed as fold basal (stimulated insulin secretion/basal insulin secretion). *$p<0.05$ by Student's t-test for +Sant7 versus IL-6 treated.

In summary, in contrast to the beneficial effects of IL-6 on α-cell function and fate (for the latter, in the presence of nutrient overload), both exogenous and endogenous IL-6 is detrimental to β-cell insulin secretory capacity. This supports the data indicating that IL-6 exacerbated nutrient overload induced β-cell apoptosis. Thus, IL-6 regulates α-cell versus β-cell fate and function in a contrasting and distinct manner.

CHAPTER 6: THE ROLE OF INTERLEUKIN-6 IN THE REGULATION OF GLUCOSE HOMEOSTASIS, AND PANCREATIC ISLET CELL MASS *IN VIVO*

6.1 BACKGROUND

Similar to obesity in humans (Herder et al., 2005; Spranger et al., 2003), HF diet increases circulating levels of IL-6 (Jiao et al., 2008). In addition, data presented in this thesis revealed increased islet derived IL-6 in response to HF feeding (Figure 2E). Therefore, *in vivo* studies using the systemic IL-6 knockout mouse were initiated to investigate the effect of obesity induced elevated IL-6 levels (systemic and local) on islet cell mass.

Interleukin-6 knockout mice have been shown to develop adult onset of obesity and glucose intolerance after 24 weeks of age, due to the absence of IL-6 signalling in the central nervous system (Wallenius et al., 2002). However, the existing literature with respect to the phenotype of these mice is contradictory, and until now no thorough investigation of pancreatic islet function and mass has been reported. Thus, these studies were initiated with the aim to gain insight into the role of IL-6 in the regulation of glucose homeostasis and pancreatic islet cell mass under HF diet induced obesity conditions.

6.2 RESULTS

6.2.1 Metabolic effects of 8 week high fat diet feeding on wild type and interleukin-6 knockout mice

At the age of 12 weeks, prior to starting the short-term HF diet study, wild type and IL-6 knockout mice display similar glucose tolerance and insulin sensitivity as determined by glucose and insulin tolerance tests respectively (Figure 22AB). Furthermore, β-cell secretory function assessed by glucose stimulated insulin secretion *in vitro*, also revealed no difference between the two genotypes (Figure 22D). In addition to similar β-cell function, there were no differences in islet morphology, % α-cell and % β-cell area/islet were similar in wild type and IL-6 knockout mice prior to starting the HF diet (Figure 22C), and as shown in figure 11C, IL-6 caused increased proliferation in islets from IL-6 knockout mice, demonstrating that these endocrine cells have the ability to replicate. These data support the data from Di Gregoria et al., which also indicate that the IL-6 knockout mice have no basal metabolic phenotype.

High fat feeding increased circulating IL-6; in wild type mice IL-6 was increased from 3.0 ± 0.9 pg/ml in chow controls to 9.1 ± 2.5 pg/ml ($p<0.05$, $n=5$) in HF diet fed mice, with no detectable IL-6 in IL-6 knockout mice. Other inflammatory cytokines and chemokines associated with obesity were also increased in response to the HF diet.

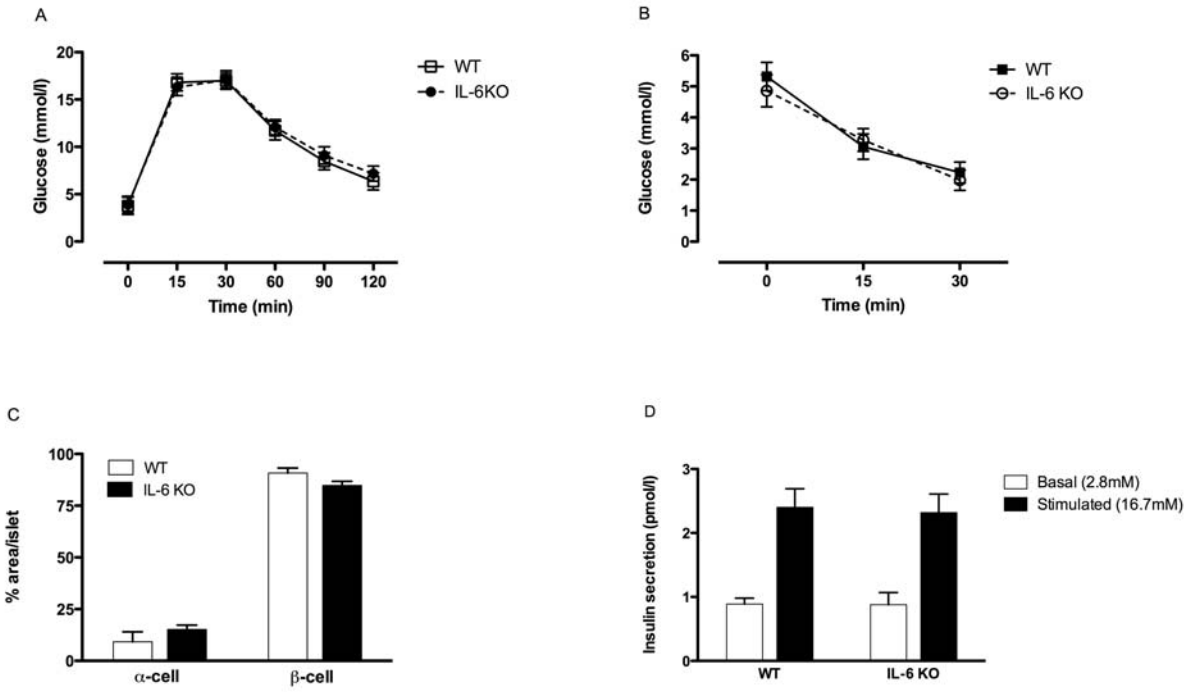

Figure 22. Glucose tolerance, insulin tolerance, islet morphology, and islet function in 10 – 12 week old wild type and IL-6 knockout mice. Intraperitoneal glucose tolerance test (ipGTT) (A), and intraperitoneal insulin tolerance test (ipITT) (B), in wild type (WT) and IL-6 knockout (IL-6 KO) mice after injection of 2g glucose/kg body weight and 0.75 mU/g body weight recombinant insulin respectively. (C) % α-cell and β-cell area/islet in wild type (WT) and IL-6 knockout (IL-6 KO) at the age of 12 weeks (n=5). (D) Glucose stimulated insulin secretion during 1 hour static incubation in KRBB containing 2.8 mM glucose (Basal) and 16.7 mM (Stimulated) in islets from wild type (WT) and IL-6 knockout mice (IL-6 KO) (n=4).

Table 1. Fasting plasma cytokines, chemokines and hormones in wild type and IL-6 knockout mice after 8 weeks on HF diet.

	CHOW		**High fat diet**	
	WT	IL-6 KO	WT	IL-6 KO
IL6 (pg/ml)	3 ± 0.9	-	9.1 ± 2.5*	-
TNF-α (pg/ml)	2 ± 0.4	1.8 ± 0.6	10.6 ± 3.9*	19.7 ± 5.5*
MCP-1 (pg/ml)	17.9 ± 1.7	13.3 ± 5.7	33.8 ± 7.1*	30.3 ± 3.7*
IL-1β (pg/ml)	11.4 ± 5.9	3.6 ± 1.0	47.6 ± 27.9	32.1 ± 15.7
KC (pg/ml)	4.6 ± 0.8	4.8 ± 1.3	13.2 ± 3.9*	7.11 ± 1.5
Insulin (ng/ml)	0.37 ± 0.02	0.39 ± 0.09	0.46 ± 0.1	0.24 ± 0.05#

* represents $p<0.05$ as determined by the Student's t-test comparing chow and HF diet (of the same genotype). For insulin, the # compares wild type (WT) and IL-6 knockout (IL-6 KO) after 8 weeks HF diet. All parameters are measured in the fasted state, (n=5).

To evaluate the role of IL-6 in regulating glucose homeostasis and islet morphometry *in vivo*, wild type and IL-6 knockout mice were challenged with a HF diet for 8 weeks. The HF diet contained 58, 26 and 16% calories from fat, carbohydrate and protein, respectively, a total of 5.6 kcal/g, whereas the control diet (chow) contained 29, 39 and 32% calories from fat, carbohydrate and protein, respectively, a total of 2.8 kcal/g.

Both wild type and IL-6 knockout mice on chow and on HF diet increased body weight similarly throughout the 8 weeks on diet, with the HF fed groups increasing significantly more than chow fed mice (Figure 23AB). There was no difference in total accumulative food intake between genotypes (Figure 23CD). After the 8 weeks of diet blood glucose levels measured after an overnight fast (12 h), and during fed conditions did not differ between genotypes. Further, 8 weeks of HF diet was not a sufficient time to cause either fasting or fed hyperglycemia (Figure 23EF).

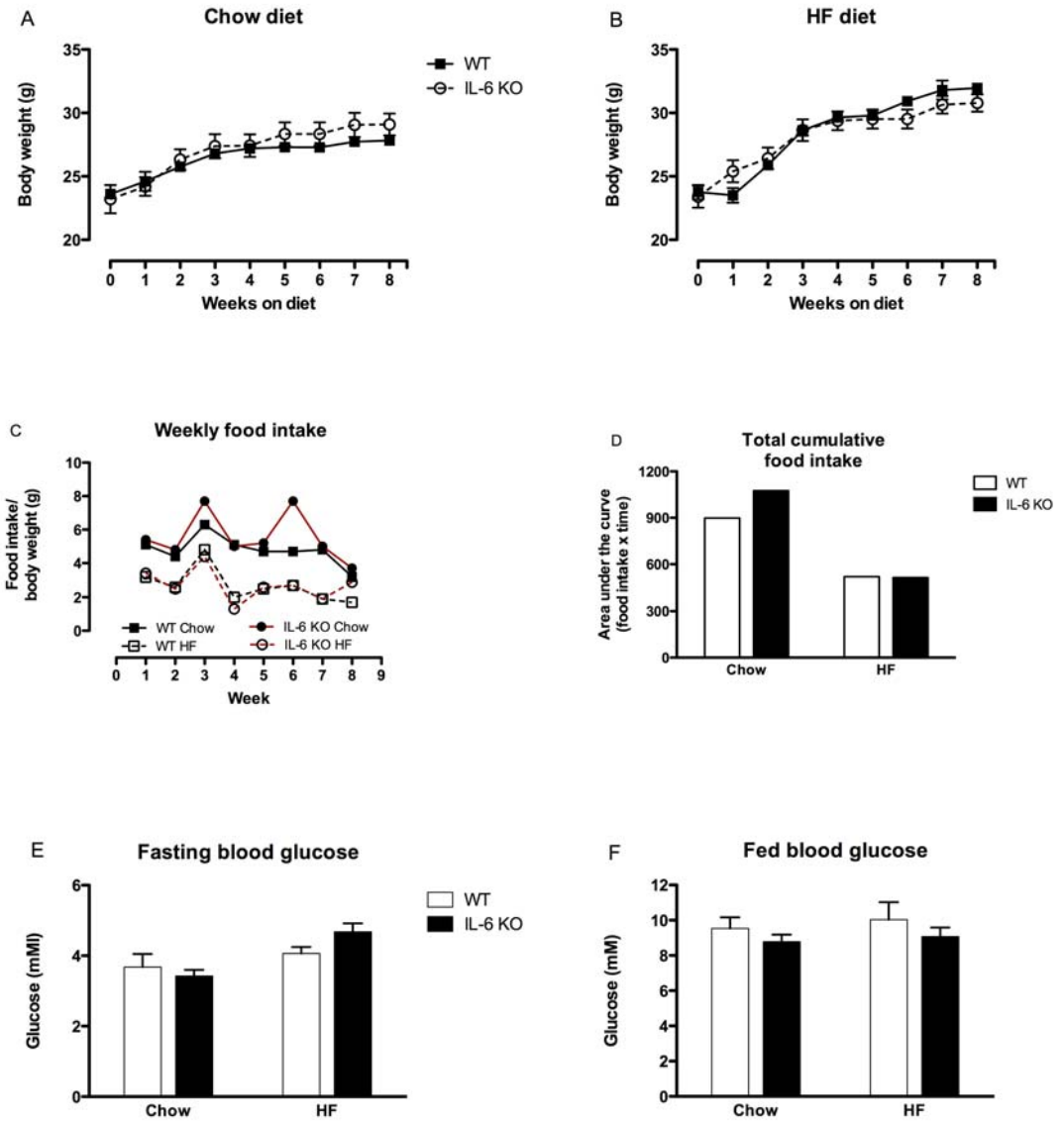

Figure 23: Body weight gain, food intake, and blood glucose in wild type and IL-6 knockout mice in response to short term HF diet feeding. Body weight gain in wild type (WT) and IL-6 knockout (IL-6 KO) mice fed a chow (A) or HF diet (B) over 8 weeks. Food intake relative to body weight determined weekly over the 8 weeks on diet in wild type and IL-6 knockout mice on chow and HF diet (C). Total cumulative food intake in wild type and IL-6 knockout mice (D). Fasting (E) and random fed (F) blood glucose levels in wild type and IL-6 knockout mice fed a HF diet for 8 weeks. For all n=5.

However, when challenging overnight fasted wild type and IL-6 knockout mice with 2 g glucose/kg bodyweight, the mice on HF diet displayed impaired glucose tolerance compared to chow fed mice at 30 and 60 minutes after glucose injection (Figure 24A-C). This effect was not

accompanied by changes in insulin sensitivity, as assessed with an insulin tolerance test using 0.75 mU/g body weight recombinant human insulin. Further, HF diet did not seem to induce insulin resistance at this time point (Figure 24D-F). At sacrifice, fasting plasma glucagon levels were not influenced by the HF diet (Figure 25A), and 8 weeks HF feeding was not sufficient to cause an increase in circulating insulin (Figure 25B), consistent with no effect on insulin sensitivity. However, IL-6 knockout mice on HF diet showed decreased circulating insulin, revealing a tendency towards improved insulin sensitivity as determined by HOMA-IR (Figure 24F).

In addition, the HF diet had no effect on pancreatic insulin content (pM insulin/mg pancreas), which also did not differ between genotypes: wild type chow: 12.2 ± 1.4; wild type HF: 8.5 ± 1.5; IL-6 knockout chow: 8.6 ± 1.4; IL-6 knockout HF: 12.7 ± 3.3 (n=5).

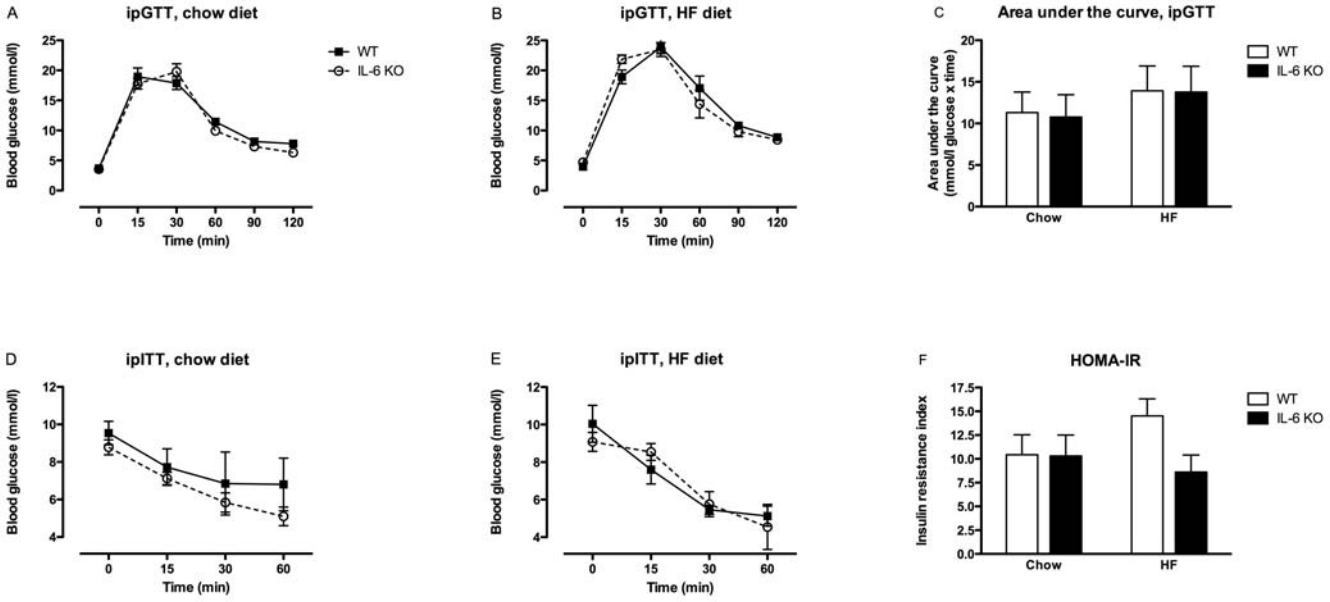

Figure 24: Regulation of glucose tolerance in wild type and IL-6 knockout mice after short term HF diet feeding. Intraperitoneal glucose tolerance test (ipGTT) (2g glucose/kg body weight) (A,B) and intraperitoneal insulin tolerance test (ipITT) (0.75 mU/g body weight recombinant insulin) after an overnight fast (D,E) in wild type (WT) and IL-6 knockout (IL-6 KO) mice fed a chow (A,D) or HF diet for 8 weeks (B,E). (C) Area under the curve for ipGTT. (F) HOMA-IR ((fasting insulin x fasting glucose)/22.5) in wild type and IL-6 knockout mice after 8 weeks diet (for all n=5).

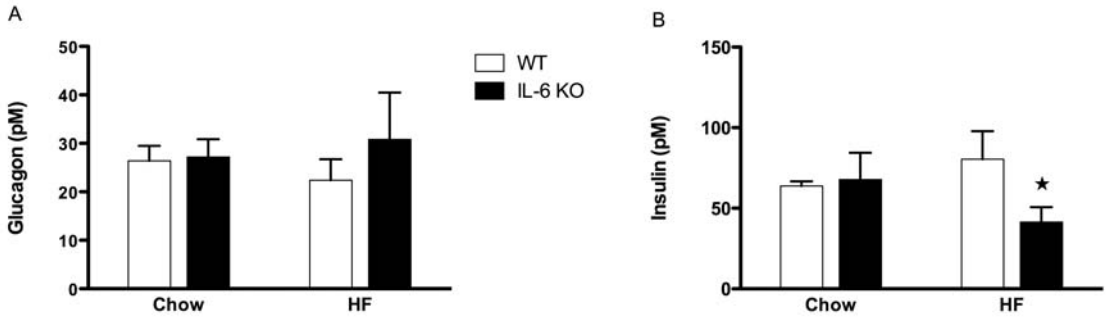

Figure 25: Fasting plasma glucagon and insulin in wild type and IL-6 knockout mice after short term HF diet feeding. Plasma glucagon (A), and insulin (B) in wild type (WT) and IL-6 knockout (IL-6 KO) mice fed a chow or HF diet for 8 weeks (For all n=5). * $p<0.05$ by Student's t-test for WT versus IL-6 knockout.

Assessment of pancreatic islet morphometry indicated no differences in islet area between genotypes or as a result of diet, however, IL-6 knockout mice tended to show smaller islets with a higher density of islets per given pancreatic section area (Figure 26B). Interestingly, there was a dramatic increase in % α-cell area/islet due to HF diet, an effect that was not present in IL-6 knockout mice. This correlated with an overall increased α-cell mass in wild type mice on HF diet compared to chow fed animals (Figure 26E). This effect was IL-6-dependent, as it was not present in IL-6 knockout mice on HF diet. Further, there were no differences between genotypes on chow diet (Figure 26E). In contrast, at this time point β-cell mass showed no differences between genotypes and due to HF diet (Figure 26F). The increase in α-cell mass due to HF diet was due to α-cell hyperplasia within individual islets, yielding a greater % α-cell area/section, since islet density and pancreas weight (mg) were unchanged. Pancreas weight (mg): wild type chow: 334 ± 26; wild type HF: 345 ± 16; IL-6 knockout chow: 334 ± 11; IL-6 knockout HF: 372 ± 21 (n=5).

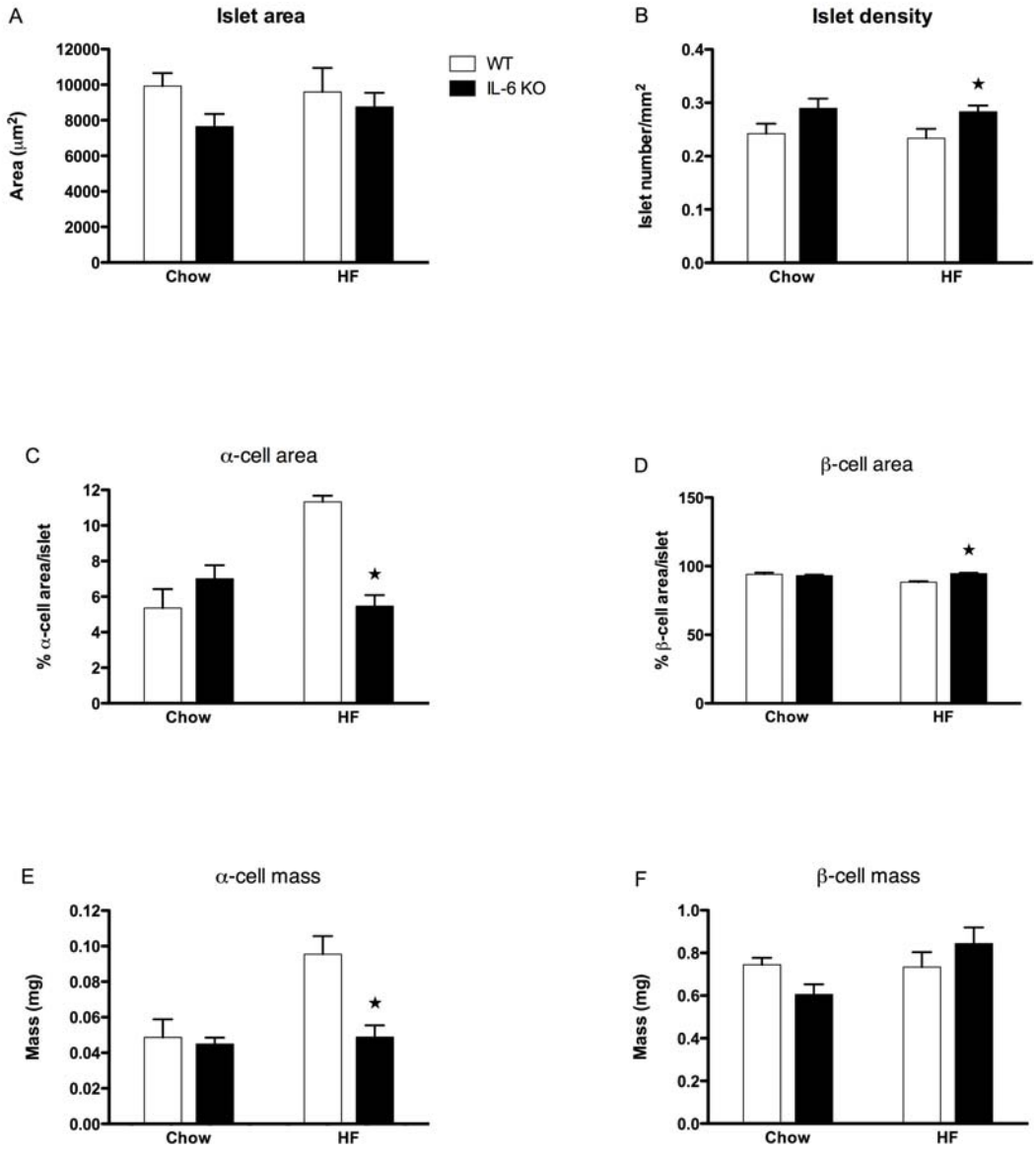

Figure 26: Interleukin-6 is required for HF diet induced increased α-cell mass. Average islet area (A), islet density (B), % α-cell area/islet (C) % β-cell area/islet (D), α-cell mass (E), and β-cell mass (F) in wild type (WT) and IL-6 knockout (IL-6 KO) mice fed a chow or HF diet for 8 weeks (for all n=5). In all cases, 3 sections per animal 200 μm apart were analyzed comprising 90-110 islets per animal. * $p<0.05$ by Student's t-test for WT versus IL-6 knockout.

Assessing proliferation with Ki67 immunohistochemistry, showed an increase in Ki67 positive α-cells/islet in wild type mice on HF diet, with no effect in the IL-6 knockout mice (Figure 27A). Strikingly, there was also a strong increase in Ki67 positive α-cells/islet in IL-6 knockout mice versus wild type animals on chow diet (Figure 27A). This supports the above *in vitro* data insomuch as IL-6 knockout α-cells are clearly able to proliferate *in vivo*. Contrary, indicative of an impaired α-cell function to the α-cell, there was a significant decrease in Ki67 positive β-cells in both wild type and IL-6 knockout mice fed a HF diet compared to chow diet feeding (Figure 27B). Similar to α-cells, IL-6 knockout mice on chow diet showed a much enhanced level of Ki67 positive β-cells, possibly

indicating a compensatory response (Figure 27B). TUNEL staining for apoptosis revealed no single apoptotic cell in 3 sections analyzed per pancreas per mouse, n=5 per group (approximately 60 – 100 islets per mouse).

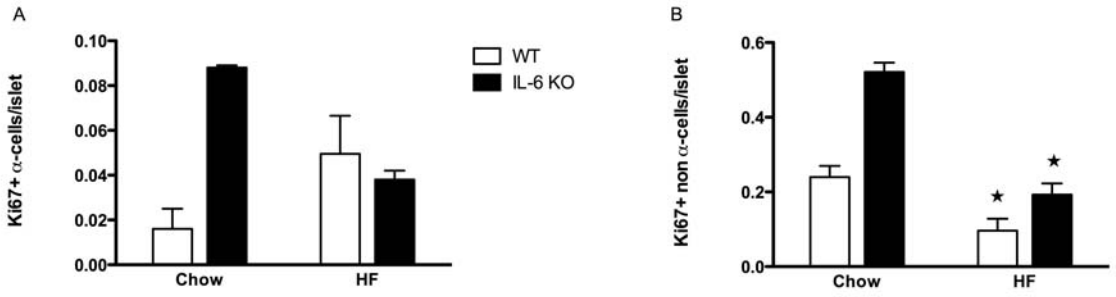

Figure 27: Regulation of α-cell and non-α-cell proliferation in wild type and IL-6 knockout mice after short term HF diet feeding. Ki67 positive α-cells (A) and non α-cells (B) in wild type (WT) and IL-6 knockout (IL-6 KO) mice after 8 weeks on chow or HF diet (for all n=5). In all cases, 3 sections per animal 200 µm apart were analyzed comprising 90 - 110 islets per animal. * $p<0.05$ by Student's t-test for HF diet versus Chow (of the same genotype).

Thus, short-term HF feeding demonstrates that IL-6 knockout mice are more insulin sensitive compared to wild type controls on HF diet, with no significant effect on glucose homeostasis. Further, 8 weeks of HF feeding led to a dramatic increase in α-cell mass in wild type mice only; demonstrating that IL-6 is required for a HF diet induced increased α-cell mass.

6.2.2 Metabolic effects of 18 week high fat diet feeding on wild type and interleukin-6 knockout mice.

To investigate if a lack of IL-6 during long-term HF diet feeding has more dramatic effects on glucose homeostasis, wild type and IL-6 knockout mice were placed on HF diet for 18 weeks. At 22 weeks of age, body weight was similar between genotypes in HF groups (Figure 28B), and systemic IL-6 was significantly elevated only in wild type mice (19.0 ± 6.8 pg/ml; n=9 vs. 99.9 ± 32.4 pg/ml, in chow versus HF respectively, n=8, $p<0.05$). While HF diet increased fasting glycemia, there were no differences between genotypes (Figure 29A). Interestingly, fed blood glucose was increased only in IL-6 knockout mice on HF diet (Figure 29B).

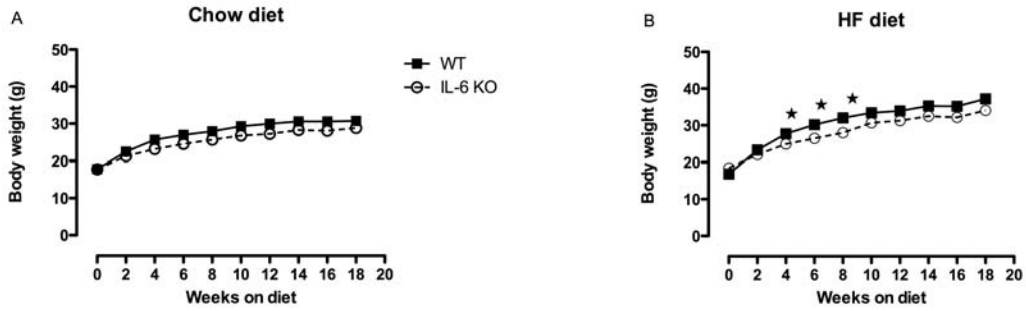

Figure 28: Body weight gain in wild type and IL-6 knockout mice on long term HF diet. Body weight in wild type (WT) and IL-6 knockout (Il-6 KO) mice fed a chow (A) or HF diet (B) for 18 weeks (each group represents n=10 animals). * $p<0.05$ by Student's t-test for comparison between genotypes.

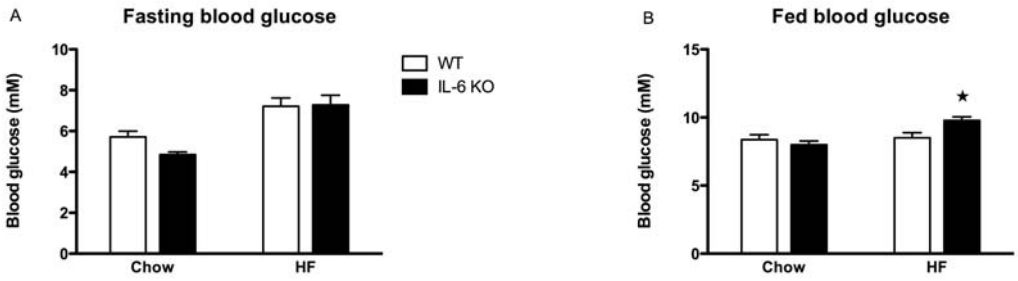

Figure 29: Fasting and fed glycemia in wild type and IL-6 knockout mice after 18 weeks of HF diet. Fasting (12 hours) blood glucose (A) and random fed blood glucose (B) levels in wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow or HF diet (each group represents n=10 animals). * $p<0.05$ by Student's t-test for comparison between genotypes.

When assessing systemic fasting glucagon and insulin levels at sacrifice, IL-6 knockout mice had paradoxically decreased hormone levels (Figure 30AB). The decrease in circulating insulin in IL-6 knockout mice on HF diet supports the 8 week HF diet data, and it follows that the HOMA-IR is also reduced in these animals (Figure 32C). In addition, there was no difference in fasting GLP-1 levels in wild type mice due to diet, however, interestingly GLP-1 levels were undetectable in IL-6 knockout mice both on chow and HF diet: (19.2 ± 4.6 pg/ml; n=7 vs. 17.1 ± 4.3 pg/ml, in chow versus HF respectively, n=6).

Next, assessment of glucose tolerance in IL-6 knockout mice revealed they were unable to clear blood glucose as rapidly as wild type mice (Figure 31B), while chow fed mice showed no differences between genotypes. This was paralleled by significantly reduced insulin secretion in HF diet fed IL-6 knockout mice during this glucose tolerance test (Figure 31D). Further, insulin sensitivity was unchanged or enhanced in IL-6 knockout HF diet fed mice, as shown by an insulin tolerance test (Figure 32B).

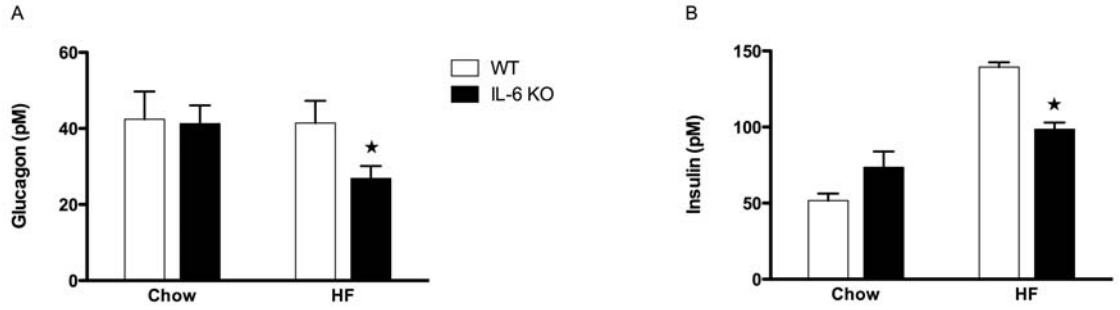

Figure 30: Fasting plasma glucagon and insulin in wild type and IL-6 knockout mice after 18 weeks of HF diet. Fasting plasma glucagon (A), and insulin (B) in wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow or HF diet. Plasma glucagon and insulin were determined by Luminex. * $p<0.05$ by Student's t-test for comparison between genotypes.

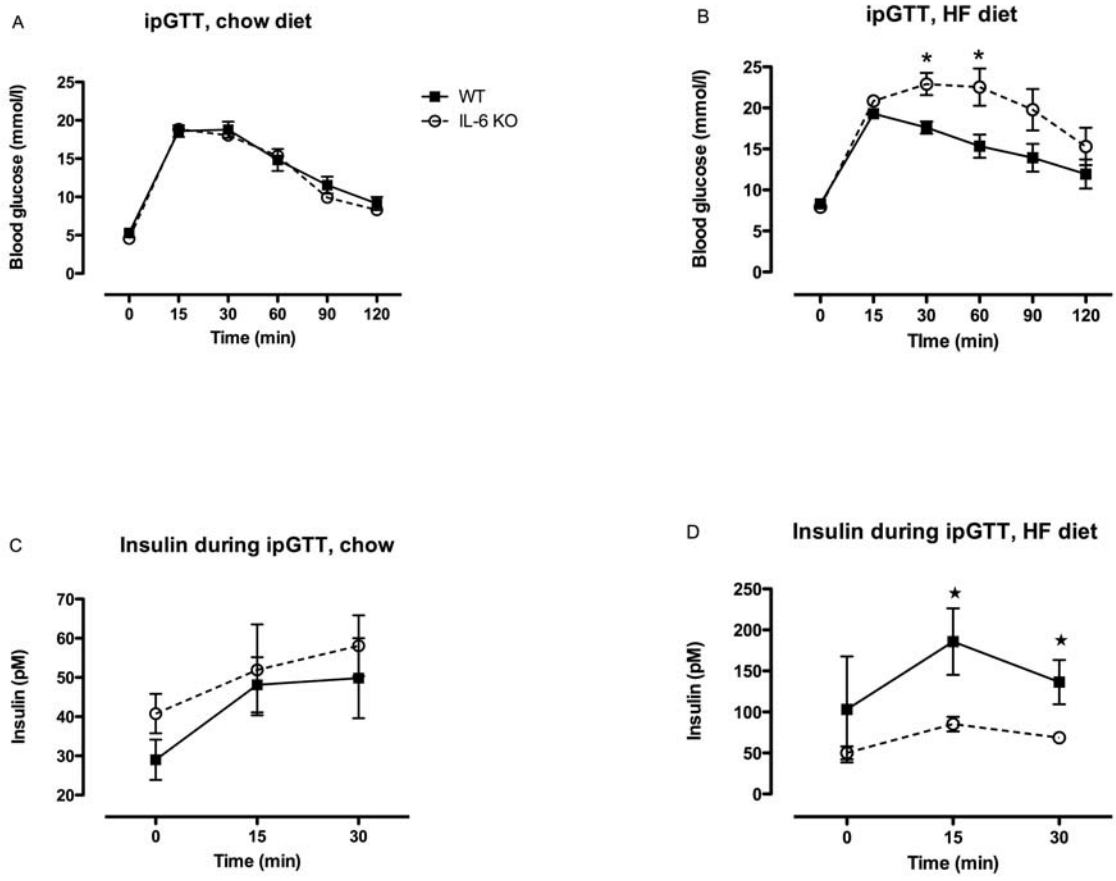

Figure 31: Glucose tolerance and insulin secretion in wild type and IL-6 knockout mice after 18 weeks of HF diet. Intraperitoneal glucose tolerence test (2g glucose/kg body weight; A, B), and insulin secretion (C,D) during this glucose challenge in 12 hour fasted wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow (A,C) and HF diet (B,D) (n=7-9). * $p<0.05$ by Student's t-test for comparison between genotypes.

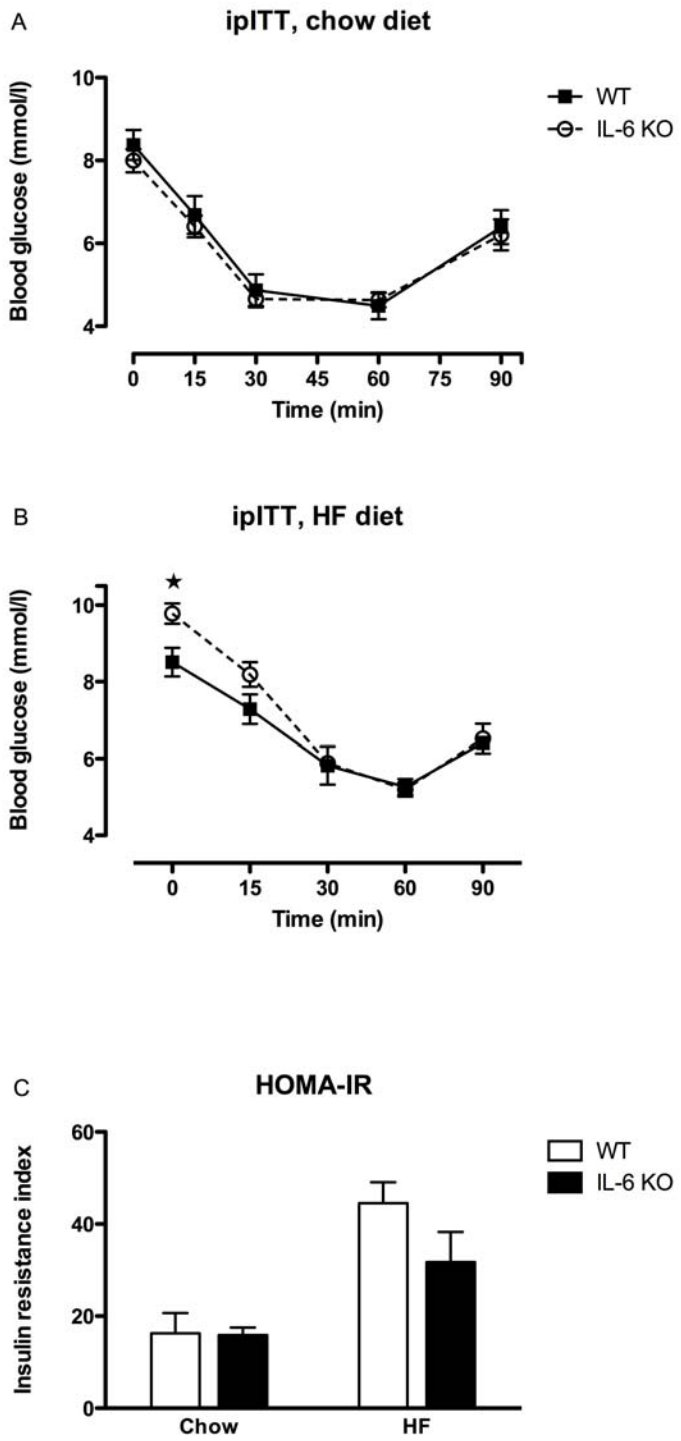

Figure 32: Insulin sensitivity in wild type and IL-6 knockout mice after 18 weeks of HF diet. Intraperitoneal insulin tolerence test in 3 hour fasted wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow (A) and HF diet (B). (C) HOMA-IR (Fasting insulin X fasting glucose) divided by 22.5) in wild type (WT) and IL-6 knockout after 18 weeks chow or HF feeding (n=7-9). * $p<0.05$ by Student's t-test for comparison between genotypes.

To summarize, despite improved insulin sensitivity as assessed by HOMA-IR and by an insulin tolerance test, IL-6 knockout mice on HF diet show impaired glucose tolerance and increased fed blood glucose levels. This could be due to dysfunctional glucagon secretion during the glucose tolereance test. However, there were no differences in circulating glucagon levels during this glucose tolerance test (Figure 33). Thus, since insulin secretion does not match blood glucose levels, we conclude that the IL-6 knockout animals on HF diet for 18 weeks show impaired β-cell function.

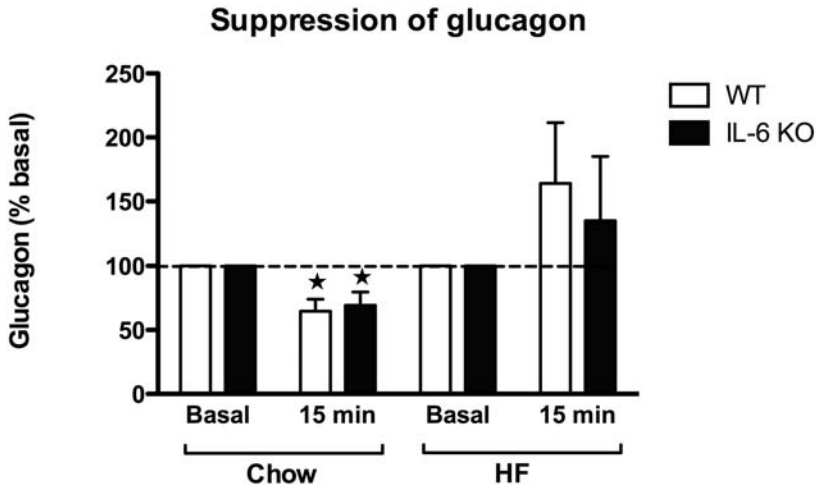

Figure 33: High fat diet impairs high glucose suppression of glucagon secretion. Fasting (12 hours) plasma glucagon (basal), and plasma glucagon 15 min after a 2g glucose/kg body weight intraperitoneal injection in wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow or HF diet (n=5-7). * $p<0.05$ by Student's t-test for comparison of 15 min to basal.

Assessment of pancreatic islet morphology indicated a trend towards increased islet size in HF fed mice with no difference between genotypes (Figure 34A); further, there was no difference in islet density between genotypes or due to diet (Figure 34B). Similar to the short-term HF diet study, islet morphology assessment showed a dramatic increase in % α-cell area/islet due to the HF diet in wild type mice only (Figure 34C). This correlated with an increased α-cell mass in wild type mice on HF diet compared to chow fed animals, while there was no difference between genotypes on chow diet (Figure 34E). In contrast, β-cell mass showed no difference between genotypes and due to the HF diet (Figure 35F). As after 8 weeks, the increase in α-cell mass due to HF diet after 18 weeks was due to α-cell hyperplasia within individual islets, yielding a greater % α-cell area/section, since islet density and pancreas mass/weight were unchanged: wild type chow: 8.7±0.2; wild type HF: 8.5±0.4; IL-6 knockout chow: 9.3±0.3; IL-6 knockout 8.7±0.3 (n=10). Furthermore, there were no differences in pancreatic insulin content (pM insulin/mg pancreas weight: wild type chow: 76.1±8.7; wild type HF: 57.7±6.1; IL-6 knockout chow: 58.9±7.1; IL-6 knockout 48.0±9.6) (n=10).

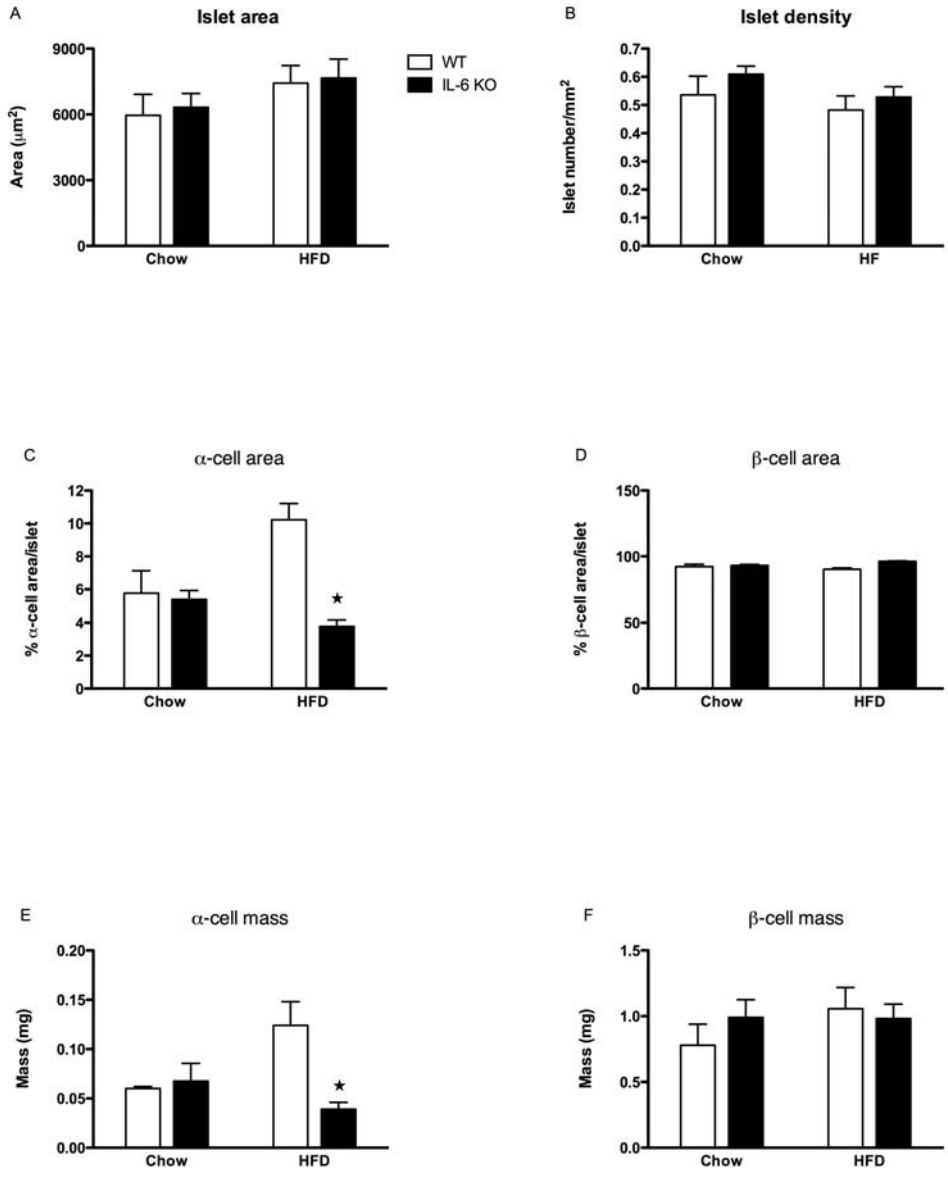

Figure 34: Lack of α-cell mass expansion in IL-6 knockout mice compared to wild type controls after 18 week HF feeding. Average islet area (A), islet density (B), % α-cell area/islet (C), % β-cell area/islet (D), α-cell mass (E), and β-cell mass (F) in wild type (WT) and IL-6 knockout (IL-6 KO) mice fed a chow or HF diet for 18 weeks (n=6-9). In all cases, 3 sections per animal 200 μm apart were analyzed comprising 90-110 islets per animal.* $p<0.05$ by Student's t-test for comparison between genotypes.

Assessing proliferation with Ki67 immunohistochemistry showed an increase in Ki67 positive α-cells in wild type mice versus IL-6 knockout on chow diet (Figure 35A). Interestingly, HF feeding caused a decrease in the number of proliferating α-cells/islet in wild type mice, whereas there was no effect of HF feeding on α-cell proliferation in IL-6 knockout mice at this time point (Figure 35A). Similar to the short-term diet, there was a tendency towards a decrease in Ki67 positive β-cells/islet in response to the HF diet, an effect that appeared to be more pronounced in the wild type mice (Figure

35B). TUNEL staining for apoptosis revealed no single apoptotic cell in 3 sections analyzed per pancreas per mouse, n=5 per group (approximately 60 - 100 islets per mouse).

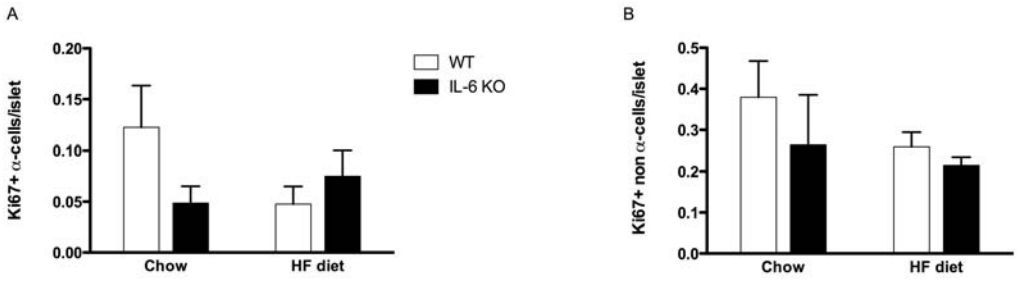

Figure 35: Pancreatic α-cell and β-cell proliferation in wild type and IL-6 knockout mice in response to 18 week HF diet feeding. Ki67 positive α-cells (A) and non α-cells (B) in wild type (WT) and IL-6 knockout (IL-6 KO) mice after 18 weeks on chow or HF diet (n=6-8). In all cases, 3 sections per animal 200 μm apart were analyzed comprising 90 - 110 islets per animal.

Therefore, long-term HF diet feeding of IL-6 knockout mice leads to reduced α-cell mass and reduced fasting glucagon levels relative to wild type mice. Paradoxically, the dominant phenotype of the IL-6 knockout mice on HF diet with respect to glucose homeostasis is a β-cell defect, resulting in reduced insulin secretion.

CHAPTER 7: DISCUSSION

The present thesis has examined the regulation of the pancreatic islet by IL-6 and identified the α-cell as a source of IL-6, and IL-6 as a regulator of the pancreatic α-cell. The pancreatic islet, specifically the α-cell, expresses a high amount of IL-6 receptor message compared to other rodent tissues. In support of α-cell specific IL-6 effects, IL-6 regulates α-cell proglucagon production, glucagon secretion, increases α-cell proliferation, and inhibits α-cell apoptosis induced by a nutritional overload in the form of elevated glucose and palmitate *in vitro*. Remarkably, IL-6 enhanced β-cell apoptosis in the presence of elevated glucose and palmitate, while effects on β-cell proliferation and β-cell function were secondary to α-cell effects kinetically *in vitro*. Further, *in vivo* data support the concept that elevated systemic IL-6 levels regulate glucagon secretion and α-cell mass. Thus, IL-6 is a positive regulator of α-cell glucagon secretion and α-cell fate, and displays distinct effects on α-cell versus β-cell fate.

7.1 NUTRITIONAL REGULATION OF ISLET DERIVED INTERLEUKIN-6 AND THE INTERLEUKIN-6 RECEPTOR *IN VITRO*

The finding that mouse and human pancreatic islets respond to a nutritional overload by increasing the release of IL-6 supports the paradigm that pancreatic islets produce cytokines and chemokines in response to metabolic stress. In addition to IL-6, IL-1β, PANDER, IL-8, G-CSF, and MIP-1α are among the islet-derived cytokines/chemokines reported to be elevated in response to a nutritional overload (Ehses et al., 2007; Maedler et al., 2002; Yang et al., 2005). The role of this islet stress response is unknown, as is its consequence for islet cell function and survival. Increased levels of cytokines and chemokines are typically associated with an inflammatory response to tissue injury, and it is tempting to speculate that the islet inflammatory response is a "call for help" to attract immune cells for regenerative purposes. On the long-term, locally produced cytokines and chemokines (islet-derived or released from invaded immune cells) may be detrimental for pancreatic islet function and survival. This work has presented evidence that islet-derived IL-6 acts in an autocrine/paracrine manner to increase islet cell proliferation and impair β-cell secretory function supporting a role for locally produced cytokines in the regulation of the pancreatic islet *in vitro*. Whether this also occurs *in vivo* remains to be investigated. Yet, the concept that a pro-inflammatory environment is detrimental to β-cell function has been proven in a recent clinical study where patients with type 2 diabetes were treated with the naturally occurring IL-1 receptor antagonist, IL-1Ra. Twelve weeks treatment caused an improved β-cell secretory function concomitant with significantly reductions in systemic IL-6 levels (Larsen et al., 2007).

Interleukin-6 is not the first cytokine to be localized to the pancreatic α-cell. Interleukin-8 and PANDER are shown to be co-localized to the α-cell and glucagon granule respectively (Cao et al.,

2003; Ehses et al., 2007); this is in contrast to IL-1β, which is found to be located in the β-cell insulin granule (Donath et al., 2005). The localization of these cytokines to hormone containing granules leads to the assumption that they are co-secreted in concert with the respective hormones. Glucagon secretion is stimulated under low glucose conditions, however, whether these conditions are also associated with elevated IL-6 was not investigated in the present study. A recent paper revealed elevated circulating IL-6 levels during hypoglycemia (Dotson et al., 2008), suggesting a possible role for glycemia in the regulation of IL-6 release from tissues. In fact, chronically elevated glucose and palmitate increased IL-6 release from human and mouse islets. These conditions of metabolic stress are known to stimulate chronic insulin and glucagon secretion, thereby providing a conduit for IL-6 release together with glucagon. However, the exact mechanism of release remains to be elucidated.

The very high islet expression of the IL-6 receptor was an interesting finding suggesting that the islet is an IL-6 sensitive tissue. However, whether the differences observed at mRNA level translate into protein expression remains to be investigated. The data moreover indicate species differences in the tissue distribution of the IL-6 receptor, as revealed by comparison of mouse and rat tissues, thus to further understand the importance and relevance of the IL-6 receptor in the pancreatic islet, human islets should be studied.

Comparison of FACS sorted rat α-cells and β-cells revealed a higher level of IL-6 receptor transcript in the α-cell enriched fraction. Comparison of the IL-6 receptor mRNA to another α-cell and β-cell housekeeping gene, ACADM (medium-chain acyl-coA dehydrogease; (Martens et al., 2007), reveals that its mRNA expression is 80 % of that of ACADM mRNA in α-cells and 60 % in β-cells, supporting the above. Interleukin-6 receptor mRNA levels in α-cells were 21, 157, 41 and 69 % and compared to mRNA for the GIP receptor (Moens et al., 1996), prolactin receptor (Sorenson and Stout, 1995), neuroserpin, and cholecystokinin A receptor (Kageyama et al., 2005) respectively (n=2), confirming the relative abundant expression of the IL-6 receptor in islet α-cells. In β-cells, IL-6 receptor mRNA levels were 6, 7, 12, and 3 % compared to these same receptors respectively (n=2). On the protein level however, western blot analysis on FACS purified fractions of these cells did not confirm the difference observed on mRNA level, and immunohistochemistry of pancreatic tissue sections revealed co-localization of the IL-6 receptor with glucagon positive cells only. Thus based on *in vivo* evidence, the IL-6 receptor is located to the α-cells, however there is substantial *in vitro* evidence favoring β-cell expression of this receptor also.

It is not clear why the IL-6 receptor was only localized to α-cells in pancreatic tissue sections given its mRNA and protein expression in isolated β-cells. A number of explanations are possible: (1) The β-cell IL-6 receptor expression may be species specific, and we only have evidence for its expression in purified RAT β-cells; (2) The technique is not sensitive enough to detect low levels of IL-6 receptor expression in β-cells in tissue sections. Only careful analysis of purified cell types *in vitro* and tissue specific IL-6 receptor knockout models will help elucidate this in the future.

Considering the difference in islet cell composition between rodent and human islets, and in particular the greater fraction of α-cells in human islets (35% α-cells in human islets versus 18% in rodent islets) (Brissova et al., 2005) it is interesting to speculate that human islets may be more IL-6 responsive as compared to rodent islets.

7.2 INTERLEUKIN-6 REGUALTION OF PANCREATIC ISLET FUNCTION *IN VITRO*

The observed effects of IL-6 on α-cell and β-cell function *in vitro* are distinct. The α-cell glucagon secretory response to low glucose is improved after pre-incubation with IL-6, whereas pre-incubation impairs glucose stimulated insulin secretion in the β-cell. Kinetically the α-cell effect is an early event (4 hours), whereas the deleterious effect of IL-6 on the β-cell is a late event (3 days). Considering IL-6 having direct effects on both α-cells and β-cells it is tempting to speculate that the early IL-6 induced increase in glucagon secretion improves β-cell glucose competence (Huypens et al., 2000), thus delaying the deleterious direct effect of IL-6 on β-cell secretory function. This hypothesis is supported by the kinetic profile of IL-6 induced glucagon secretion, where the early increase is followed by a significant decrease in glucagon secretion after 4 days (Figure 8B), thus approximately at the same time as the observed impairment in β-cell function. Another very attractive hypothesis is that the kinetic delay in the deleterious effect on β-cell secretory function is due to increased GLP-1 being released from the α-cell acting on the β-cell to improve its secretory function. Since the proglucagon gene gives rise to both glucagon and GLP-1, the observed IL-6 induced increase in proglucagon mRNA may lead to increased glucagon and/or GLP-1 protein requiring the presence of PC2 and PC1/3 respectively. However, the absence of an IL-6 effect on arginine stimulated glucagon secretion and glucagon content suggests that the increase in glucagon secretion is not a consequence of increased glucagon production.

The hypothesis that IL-6 is involved in the regulation of α-cell derived GLP-1 is supported by the fact that the IL-6 knockout mice have no measurable levels of plasma GLP-1, and furthermore, that α-cell expression of PC1/3 seems to be dependent on IL-6 since PC1/3 was not detected in the α-cell in HF fed IL-6 knockout mice (preliminary data, not shown).

Thus overall, it seems that IL-6 is causing a beneficial environment for α-cell secretory function, evidenced by an improved glucagon secretory response under low glucose conditions, without increasing glucagon production and storage. The increase in plasma glucagon observed in response to a bolus injection of IL-6 only when mice were in a fasting state, supports a proposed physiological role for IL-6 in the regulation of glucagon secretion *in vivo*. This observation is in agreement with studies demonstrating an increase in circulating glucagon in response to IL-6 infusion in healthy humans (Hiscock et al., 2005; Tsigos et al., 1997).

The present work has furthermore provided substantial evidence for a detrimental effect of IL-6 on β-cell insulin secretory function *in vitro*. This is in agreement with previously reported studies in

rodents (Kristiansen and Mandrup-Poulsen, 2005), however, in contrast to previous data reported on human islets that showed no effect (Eizirik et al., 1994). A likely explanation for this discrepancy is the short-term incubation used in the study by Eizirik et al. and the use of ECM coated dishes by us. Further support for a detrimental role of IL-6 on β-cell function in human islets is provided by the negative correlation between basal IL-6 release from human islets and their ability to secrete insulin in response to glucose.

The present work has not provided any evidence for a direct role of IL-6 on β-cell function *in vivo*.

7.3 INTERLEUKIN-6 REGUALTION OF PANCREATIC ISLET SURVIVAL *IN VITRO*

The mitogenic and growth promoting effects of IL-6 have never been investigated in pancreatic islet cells *in vitro*. However, the RIP-IL-6 mice, specifically overexpressing IL-6 in the pancreatic β-cells revealed enlarged islets, mitotic figures, and signs of neogenesis, suggesting a proliferative and growth enhancing role of IL-6 also in the pancreatic islet *in vivo* (Campbell et al., 1994). These RIP-IL-6 mice demonstrated normal β-cell function, which is surprising when considering the present data.

There are a wealth of studies on β-cells showing various growth promotive factors that signal through STAT3 (Garcia-Ocana et al., 2000; Nielsen et al., 2001; Yamamoto et al., 2000), substantiating the potential importance of this signalling pathway for the maintenance and expansion of islet mass. In support hereof, β-cells transfected with constitutively activated STAT3 revealed increased islet cell proliferation (Tsukiyama et al., 2006), whereas β-cell specific STAT3 knockout mice display disturbed islet architecture with α-cells scattered throughout the islet (Gorogawa et al., 2004). However, a thorough investigation of islet morphometry in these mice was not performed.

Furthermore, antiapoptotic effects of IL-6 are also mediated through STAT3, and have been shown to be coupled to the induction of the anti-apoptotic genes Bcl-2 and Bcl-xL in a pro-B cell line (Fukada et al., 1996). Prosurvival effects of IL-6 have been demonstrated in mouse islets and MIN-6 β-cells, and the authors report that cytokine induced β-cell apoptosis was prevented by IL-6 (Choi et al., 2004). This is in contrast to the observed β-cell apoptotic effect of IL-6 in the present work. Choi et al. claim a protective effect of IL-6 on β-cells without distinguishing between the various islet cells. However, the increased viability is associated with increase Bcl-xL expression after 24 hours. Based on the current work, it is tempting to speculate that the prosurvival effect reported by Coi et al. is primarily due to increased α-cell viability. Supporting this hypothesis, is the short-term (24 hours) IL-6 incubation, which in our hands does not induce β-cell apoptosis, and also increases an anti-apoptotic gene Bcl-2. Another likely important reason for the distinct effects in these two studies is the use of ECM dishes in ours.

In other non-islet cell types the proliferative and growth enhancing effects of IL-6 are mediated through STAT3, and have been associated with an upregulation of cyclin D1, D2, D3, A, CDC25A, and c-myc, with concomitant down regulation of p21 and p27 (Fukada et al., 1996; Serrano et al., 2008). It has been proposed that IL-6 induced signalling leads to distinct biological outcomes that are not solely dependent on the pathway activated in the individual cell, however, that the metabolic state of the cell plays a role for the biological outcome (Kamimura et al., 2003). The finding that IL-6 protects α-cells from nutrient induced apoptosis, and further enhances apoptosis in β-cells favors the above mentioned hypothesis. These studies furthermore revealed that α-cells are more sensitive to nutrient induced apoptosis, which leads to the speculation that the culture conditions and/or the isolation procedure renders α-cells more susceptible to detrimental stimuli. In support of the distinct effect of IL-6 on nutrient induced apoptosis, is the differential effect of IL-6 on apoptosis alone. Interleukin-6 did not induce α-cell apoptosis, neither short-term or long-term, whereas there was a 12-fold induction in % apoptotic β-cells after 4 days culture.

The observation that IL-6 induced islet cell proliferation was consistent between α-cells and β-cells, however α-cell proliferation occurred prior to β-cell proliferation, which led to the hypothesis that β-cell proliferation could be an indirect effect possibly mediated through α-cell glucagon. In support of this, exogenous glucagon increased islet cell proliferation, however to a less extent than IL-6. Important to note is the ability of human and rodent islet cells to proliferate *in vitro*, this is possibly due to the ECM coated dishes creating a favorable environment for the islets allowing them to attach and spread, improving nutrient access to all cells. Interleukin-6 caused a 12-fold induction in β-cell apoptosis, however, it is important to note that these experiments were performed on dispersed mouse islet cells, which may be more prone to apoptotic stimuli in comparison to intact islets. The fact that all experiments evaluating the effect of IL-6 on proliferation were performed on whole islets (islets dispersed at the end of the treatment) it is not possible to compare the apoptotic response with the proliferative, and thus concluding whether IL-6 causes cell death or replication. However, the phenonema that proliferating cells are more prone to death is known from cancerous cells where increased apoptosis is a common observation. In addition, TUNEL staining does not distinguish between DNA repair and fragmentation, considering DNA repair after cell replication, the increased number of TUNEL positive cells could also indicate cells that recently divided. In support of an increased proliferative effect of IL-6, there was an early increase in c-myc, and decrease in the cyclin dependent kinase inhibitor p27. These early changes indicative of ongoing proliferation are likely associated with the early increased α-cell proliferation observed in mouse islets, the mild effect may be explained by the low number of α-cells relative to β-cells.

Overall, evaluating potential genes involved in proliferation and survival in mouse islets revealed only minor effects, and as already mentioned this may be explained by the low number of α-cells relative to β-cells. Indeed, a kinetic profile of IL-6 induced Bcl-2 revealed an early upregulation, followed by a down regulation after 48 hours and onwards. This profile correlates kinetically with the

initial protective effect of IL-6 on α-cell apoptosis, followed by significant β-cell apoptosis occurring thereafter. It will be interesting to evaluate IL-6-stimulated cell type specific signalling mechanisms in purified α-cell and β-cells in the future.

7.3 INTERLEUKIN-6 REGULATION OF GLUCOSE HOMEOSTASIS AND ISLET MASS *IN VIVO*

In order to investigate islet IL-6 effects in the context of metabolic stress *in vivo*, short term and long term HF diet feeding of wild type and IL-6 knockout mice was performed. The systemic IL-6 knockout mouse was created in 1994, and was reported to present with impaired immune and acute-phase responses (Kopf et al., 1994). Given the pleiotropic nature of IL-6, the IL-6 knockout mice have been used in a variety of studies with respect to whole body metabolism, revealing impaired liver regeneration (Wallenius et al., 2001), reduced stress- and cold- induced increase in energy expenditure (Wernstedt et al., 2006), and reduced exercise endurance (Faldt et al., 2004). Interestingly, the IL-6 knockout mice have been shown to develop mature onset of obesity, presumably due to lack of IL-6 actions on the central nervous system (CNS) (Wallenius et al., 2002), however these observations remain to be verified (Di Gregorio et al., 2004) and may be related to genetic background, and breeding environment. Importantly, the mature onset of obesity presented itself as increased body weight relative to wild type mice at the age of 24 weeks. Therefore, all studies reported in this thesis used IL-6 knockout mice no older than 22 weeks.

At the age of 12 weeks, prior to starting the short-term HF diet study, IL-6 knockout and wild type mice displayed no differences in glucose tolerance, insulin sensitivity, or islet morphology. Furthermore, IL-6 knockout islets displayed normal insulin secretion in response to glucose and showed normal proliferative responses to IL-6 *in vitro*. Thus, IL-6 does not appear to be necessary for normal α-cell or β-cell development and function.

In the present study body weight increased similarly in wild type and IL-6 knockout on chow and HF diet respectively, which is in agreement with previous studies on these mice at this age (Di Gregorio et al., 2004). In line with obesity in humans, systemic indicators of an ongoing inflammatory process (IL-6, TNFα, KC, MCP-1 and a tendency for IL-1β) were elevated in the circulation in response to HF feeding. The influence of elevated IL-6 levels on pancreatic α-cell fate was already apparent after 8 weeks of HF feeding. In contrast to chow fed animals displaying normal islet morphology, IL-6 knockout mice on HF diet were unable to increase their α-cell mass in response to HF diet feeding, likely due to the absence of proliferative and/or protective effects of IL-6 on the α-cell. High fat diet feeding increases circulating free fatty acids such as palmitate in addition to elevating systemic glucose. Thus, elevated IL-6 levels during HF diet feeding may stimulate α-cell proliferation and prevent glucolipotoxicity-induced α-cell apoptosis. Therefore, expansion of α-cell mass in response to HF diet feeding is IL-6-dependent *in vivo*, supporting the *in vitro* data and

suggesting α-cell-specific IL-6 effects. Also interesting to note, is the appearance of an increased α-cell mass in response to HF feeding as an early morphological event detectable prior to any change in β-cell mass. The absence of an expansion of β-cell mass in response to HF feeding in this study is probably multifactorial, where the most important factors are the negligible effect on peripheral insulin resistance by the HF diet, and the diet itself. The macronutrient composition of the HF diet is known to play an important role for the various parameters associated with a nutritional overload. However, in the absence of an increase in β-cell mass, α-cell hyperplasia rather than hypertrophy was responsible for the increase in α-cell mass. Whether increased proliferation and/or decreased apoptosis contributed to the observed α-cell mass expansion is unclear. Using Ki67 as a marker for proliferation only provides a snapshot (half-life of Ki67 has been estimated to 60 – 90 minutes) (Meer et al., 2003) of the proliferative activity, and revealed no effect of HF feeding, however it is not possible to exclude the possibility that there has been an increase in α-cell proliferation prior to the time point examined. In addition to increased proliferation, a decrease in apoptosis could contribute to the increase in α-cell mass. However, detection of apoptosis by TUNEL staining *in vivo* is notoriously difficult due to the rapid clearance of apoptotic cells by immune cells. In line with this, there was no single TUNEL positive islet cell detected in 3 sections analyzed per pancreas per mouse, n=5 per group (approximately 60 – 100 islets per mouse). Thus, these studies do not allow for a conclusive statement on whether the expansion of α-cell mass was due to increased proliferation and/or decreased apoptosis. However, based on the *in vitro* data, it can be speculated that either, or both have contributed to the increased α-cell mass. *In vitro* IL-6 was found to both induce α-cell proliferation, and to protect α-cells from nutrient induced apoptosis.

Strikingly, there was a strong increase in Ki67 positive α-cells and β-cells/islet in IL-6 knockout mice versus wild type animals on chow diet in the short-term study. Due to the proliferative role of IL-6, this was a surprising observation, indicating a compensatory proliferative response in the absence of IL-6. In the long-term study there was no difference in β-cell proliferation between genotypes, whereas the IL-6 knockout on chow diet demonstrated reduced α-cell proliferation versus wild type. This is likely due to the absence of the proliferative and anti-apoptotic effect of IL-6. Overall, the assessment of proliferation *in vivo* by Ki67 has not provided much informative data, this is possibly due to the short half-life of Ki67, in addition to the few α-cells present in mouse islets. Thus in future experiments, BrdU incoporation will be used to allow continuous assessment of islet cell proliferation.

After long-term 18 week HF diet feeding IL-6 knockout animals presented with decreased fasting glucagon levels, while exhibiting fed hyperglycemia and decreased insulin secretion in response to glucose compared to wild type controls. This is suggestive of β-cell failure, in the absence of differences in insulin resistance between genotypes. The fact that insulin sensitivity is similar in wild type and IL-6 knockout mice confirms previously reported results on these mice (Di Gregorio et al., 2004), and indicates that the absence of IL-6 does not influence peripheral insulin sensitivity,

despite the fact that IL-6 is an insulin sensitizer in skeletal muscle. The insulin sensitizing effect of IL-6 in skeletal muscle led to the hypothesis that the IL-6 knockout mice would show increased insulin resistance, however this effect might have been opposed by improved hepatic insulin sensitivity in the absence of IL-6. Thus, opposing effects of IL-6 on insulin sensitivity in skeletal muscle and liver possibly explain the similar insulin sensitivity in wild type and IL-6 knockout mice. It will be interesting to perform euglycemic hyperinsulinemic clamps in these animals on HF diet to exactly determine tissue specific insulin sensitivity.

The absence of functional β-cell compensation in IL-6 knockout mice in response to HF feeding is in agreement with previously reported data (Di Gregorio et al., 2004), however this result disagrees with the *in vitro* impairment of β-cell secretory function in response to IL-6. Thus, it can be speculated that the observed α-cell phenotype predominates a milder β-cell phenotype, or that β-cell secretory function is not directly regulated by IL-6 *in vivo*. Further, there was no defect in β-cell mass in IL-6 knockout mice, suggesting that α-cell mass expansion and glucagon may regulate β-cell secretory function. *In vitro,* we found that IL-6 increases glucagon expression in addition to regulating α-cell fate. It is known that the pancreatic α-cell helps to maintain β-cell glucose-competence via glucagon (Huypens et al., 2000), and the glucagon receptor knockout mice has impaired β-cell function (Sorensen et al., 2006). Recently, establishment of a β-cell over expressing glucagon receptor transgenic mouse confirmed this paradigm, since these mice have improved glucose tolerance and increased insulin secretion in response to glucose (Winzell et al., 2007). Thus, the data suggest that β-cell glucose-competence is impaired in IL-6 knockout animals due to reduced α-cell derived glucagon regulating the pancreatic β-cell. However, the possibility that other α-cell derived factors could also be contributing to this lack of β-cell glucose-competence cannot be excluded. Due to α-cell mass expansion being an early event kinetically, it is tempting to speculate that this is an adaptive mechanism aiming to maintain β-cell function under circumstances with increased demand.

An interesting and likely candidate of other α-cell derived factors contributing to the lack of functional β-cell compensation is GLP-1. As already mentioned, the present work has provided preliminary evidence for a role of IL-6 in regulating PC1/3. Under normal conditions PC1/3 expression within the pancreas is limited to the β-cells, however, the concept that α-cell-derived GLP-1, and thus α-cell expression of PC1/3, may have a role in β-cell survival exists, and is supported by a recent study showing enhanced islet survival after transplantation when α-cells overexpressed PC1/3 leading to increased production of GLP-1 (Wideman et al., 2007). In addition, PC1/3 expression in α-cells has been observed in rats given STZ resulting in significant increases in the levels of bioactive GLP-1 in the rat pancreas (Nie et al., 2000). Based on these studies and our preliminary observation that PC1/3 is expressed in the α-cell in the wild type mice on HF diet, it can be hypothesized that IL-6 induced α-cell derived GLP-1 (in addition to glucagon) is a mechanism by which the α-cell helps to maintain β-cell glucose competence. Supporting this hypothesis is the impaired β-cell glucose

competence and the absence of PC1/3 expression in the α-cells in IL-6 knockout mice on HF diet. This is an extremely interesting observation since it indicates a role for IL-6 in regulating PC1/3 in the α-cells and thus GLP-1 processing in these cells. Supporting this observation is the lack of measurable plasma GLP-1 in IL-6 knockout mice.

Indicative of an impaired α-cell function, postprandial suppression of glucagon is impaired in patients with diabetes (Dunning and Gerich, 2007). This phenomenon was investigated *in vivo* in mice by measuring plasma glucagon in response to a glucose challenge. Interestingly, HF diet led to a failure to suppress glucagon, however, this effect seemed to be IL-6 independent since there was no difference between genotypes.

A caveat of the present study lies in the use of a systemic ligand knockout (the IL-6 knockout), and thus a system where IL-6 signalling is absent in all tissues. Using this mouse model it is not possible to conclude that the observed phenotype is due to direct effects on the pancreatic islet cells. The data is correlative, however the *in vitro* data strongly supports a direct role for IL-6 in pancreatic islet function and survival. To obtain proof for a direct role of IL-6 on these islet cells, cell specific deletion of IL-6 signalling is required. The milder β-cell phenotype observed *in vivo* is however not supported by *in vitro* data, and this phenotype is (as discussed above) likely to be explained by the α-cell phenotype. Thus, the IL-6 whole body knockout mouse model does not allow for a conclusive statement on IL-6 not playing a role in regulating the β-cell *in vivo*. Due to the presence of a soluble IL-6 receptor that has the ability to act as an agonist in the presence of membrane bound gp130, tissue specific deletion of the IL-6 receptor is futile. Consequently, there exists no way to knockout IL-6 signalling specifically in one tissue only *in vivo*. Thus, the only way to delete IL-6 signaling in a tissue specific manner, is to delete the membrane bound gp130 signal transducer. However, the obvious caveat is related to gp130 not being a co-receptor/signal transducer for the IL-6 receptor exclusively, as it also serves as signaling transducer for LIF, OSM, CNTF, interleukin 11, etc. Future studies are planned to investigate the role of gp130 receptor signaling in both the α-cell and the β-cell using tissue specific genetic deletion (see Future directions below).

Overall, IL-6 exerts its effects on a number of metabolically active tissues, and the topic of IL-6 as a "good-guy" or "bad-guy" is a matter of ongoing debate (Mooney, 2007; Pedersen and Febbraio, 2007). Contributing substantially to the controversy is the relative recent realization that IL-6 is produced and released from the working skeletal muscle during exercise, suggestive of a beneficial role of IL-6, which is contradictory to the seemingly negative role of IL-6 when associated with elevated levels in obesity, and furthermore, the finding that chronically elevated IL-6 levels predict disease development. Since the definition of IL-6 as a myokine and the suggestion that IL-6 may be the "exercise factor", the primary target tissue of exercise induced IL-6 remains to be elucidated. A very recent study however, demonstrated a role for endogenous IL-6 acting in an autocrine manner to induce proliferation and hypertrophy of satellite cells (Serrano et al., 2008), providing one possible role of exercise induced IL-6. In addition, exercise induced IL-6 could also act as the insulin sensitizer

in skeletal muscle, thereby possibly contributing to the beneficial effects of exercise. However, re-evaluating the role of exercise induced IL-6 in the context of the data presented in the present thesis, leads to the speculation that IL-6 released from exercising muscle may act on the pancreatic α-cell to induce glucagon secretion, thereby promoting substrate availability through glucagon actions on the liver. The hypothesis that IL-6 may act as a substrate sensor and a mobiliser of substrate availability is supported by the fact that exercise induced IL-6 release from skeletal muscle is increased when the working muscle has been depleted of glycogen prior to the exercise bout (Febbraio et al., 2003). Alternatively, IL-6 actions on the α-cell could induce GLP-1, a potential mechanism by how exercise improves β-cell function. Furthermore, and purely speculative, if an increased α-cell mass is an adaptation to exercise it could be due to exercise induced IL-6, and could explain the ability to maintain circulating glucagon levels at the end of long-term physical activity in the trained versus untrained individuals.

In general, whether IL-6 is a good or a bad guy from an islet point of view seems to have more than one answer. Based on the present work, increased IL-6 is beneficial for the α-cell and deleterious for the β-cell. With respect to the contribution of IL-6 to type 2 diabetes pathophysiology, the overall consequences of tissue-specific IL-6 effects need to be considered.

The present thesis was undertaken to elucidate whether IL-6 plays a role in the regulation of the pancreatic endocrine islet. The work presented proposes that elevated IL-6 levels during obesity drive α-cell mass expansion and glucagon expression, an early event that may be required for functional β-cell compensation in response to HF diet induced insulin resistance. However, whereas increased IL-6 levels at an early stage seem to be beneficial, prolonged elevated IL-6 levels may lead to pathological glucagon secretion observed at onset and during progression of diabetes (schematic illustration figure 36). It is hoped that this thesis has provided some useful insight into the potential relevance of the elevated IL-6 levels observed in obesity and type 2 diabetes with respect to the pancreatic islet, and furthermore, that this knowledge will contribute to the basis on which decisions are made when clinical intervention is targeted at IL-6 signaling.

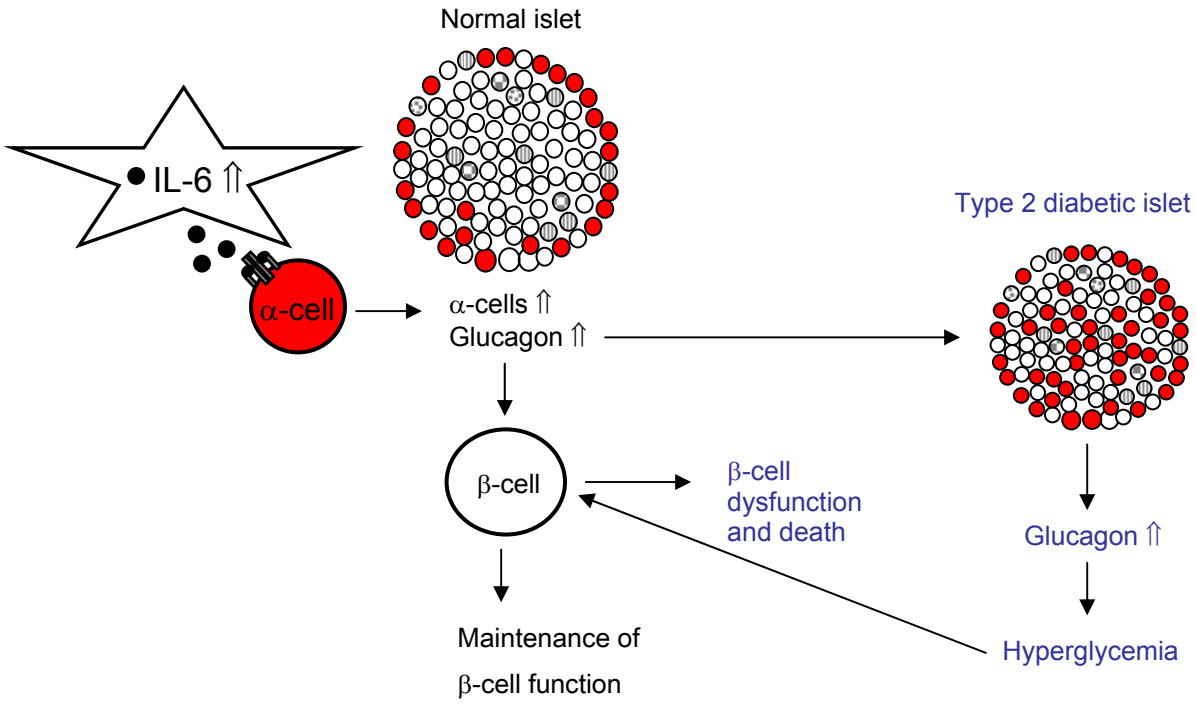

Figure 36: Elevated IL-6 levels in obesity may drive α-cell mass expansion and glucagon expression, that may be required for functional β-cell compensation in response to HF diet induced insulin resistance. However, prolonged elevated IL-6 levels may lead to pathological glucagon secretion observed at onset and during progression of diabetes, thus contributing to hyperglycemia.

The main conclusions from this thesis can be listed as follows:

1. A nutritional overload (metabolic stress) increases pancreatic islet IL-6 release.
2. IL-6 is localized to the pancreatic α-cell glucagon granule in human islets.
3. The IL-6 receptor is highly expressed in the pancreatic islet, with highest expression in the α-cell.
4. In the pancreatic islet, the IL-6 receptor is coupled to the STAT3 signalling pathway.
5. IL-6 regulates proglucagon mRNA and glucagon secretion.
6. IL-6 induces islet cell proliferation, with α-cell proliferation occurring prior to β-cell proliferation.
7. IL-6 protects α-cells from nutrient induced apoptosis, while further enhancing β-cell apoptosis.
8. IL-6 is necessary for a HF diet induced increased α-cell mass *in vivo*.
9. IL-6 knockout mice on long term HF diet are glucose intolerant due to lack functional β-cell compensation.

During the course of this thesis research, the concept that type 2 diabetes is an inflammatory disease also at the level of the pancreatic islet has been promoted and substantially supported by our laboratory in collaboration with others. Studies performed in parallel to this thesis have revealed that human islets from patients with type 2 diabetes are associated with increased numbers of macrophages (Ehses et al., 2007), that the expression of the proinflammatory cytokine IL-1β is increased in human pancreatic β-cells in patients with type 2 diabetes (Böni, 2007), that antagonism of IL-1 improves glycemia by improving pancreatic β-cell insulin secretion, while concomitantly strongly reducing circulating IL-6 (Larsen et al., 2007), and that antagonism of IL-1 reverses islet inflammation and hyperglyemia in a rodent model of type 2 diabetes (Ehses, 2008). Taken together these studies considerably strengthen the notion that type 2 diabetes is an inflammatory disease also at the level of the islet. Emphasizing the concept of an islet inflammatory state, work presented in this thesis revealed that pancreatic islets respond to a nutritional overload (metabolic stress) by increasing the release of IL-6 and various other cytokines and chemokines (Ehses et al., 2007), and furthermore provided *in vitro* evidence that locally produced IL-6 induces islet cell proliferation, and is detrimental to β-cell secretory function. Whether locally produced IL-6 is relevant for islet function and survival *in vivo* is an intriguing and potentially important question that remains unanswered. In order to address this question, local IL-6 sources have to be disrupted. Considering that both the endocrine α-cells and the islet endothelial cells are the main sources for locally produced IL-6, this is a challenging question to answer. α-Cell specific deletion of the IL-6 gene is possible, however, how to target the islet endothelial cells without influencing whole body endothelial IL-6 production would require an islet

endothelial marker only specific to the islet endothelial cells. Ideally, deletion of the IL-6 gene from these two sources would provide an excellent model to study the influence of systemic versus local production of IL-6 on pancreatic islet function and survival under conditions of a nutritional overload and during the progression to diabetes.

Results have been presented implicating a potential physiological role for IL-6 in regulating glucagon secretion under low glucose conditions, an effect possibly mediated by the increased circulating levels of IL-6 (Dotson et al., 2008) and an upregulation of IL-6 receptor expression under these conditions. Generation of an α-cell specific gp130 receptor knockout mice (currently underway) will provide an excellent model to investigate this proposed physiological role of IL-6. Whether IL-6 plays a role in the dysregulation of glucagon secretion observed in patients with diabetes would be an interesting question to answer. Interleukin-6 treatment of human islets caused an increased glucagon secretory response under both low and high glucose conditions resembling the increased glucagon secretion (and lack of suppression) observed in patients with type 2 diabetes in response to food intake. Thus, it will be interesting to further investigate the role of IL-6 on α-cell secretory function in HF diet challenged mice with a targeted deletion of gp130 in the α-cell. These studies could be complimented by studies in the RIP-IL-6 mice providing a model with excess local IL-6, and finally using the IL-6 knockout with and without IL-6 infusion could lend support to this hypothesis.

Elucidation of distinct effects of IL-6 on both basal apoptosis and nutrient induced α-cell and β-cell apoptosis was an unexpected and intriguing observation. This raises the question: does it make physiological sense for IL-6 to be anti-apoptotic in the pancreatic α-cell, while being pro-apoptotic in the β-cell? IL-6 is known to posses both pro-survival and anti-apoptotic potential, however that IL-6 is capable of inducing completely distinct effects on two neighboring cell types is an extremely fascinating finding supporting the proposed "signalling orchestrating concept". According to this concept, the distinct biological outcome depends on the signalling pathway that prevails in the individual cell type, whereas the balance between the signalling pathways activated may further depend on the metabolic state of the cell and on the combination of other stimuli. Along with this notion, it will be interesting to further investigate whether the distinct effect of IL-6 on nutrient induced α-cell and β-cell is a consequence of a different metabolic response to glucose and fatty acids (palmitate) in these two cell types. Based on the work presented in the present thesis, high glucose and palmitate induce a much enhanced apoptotic stimulus in the α-cell versus the β-cell, indicating different handling of a nutritional overload (metabolic stress) in these cells. To investigate whether the protective effect of IL-6 is specific for the given cell, or related to the metabolic handling of a nutritional overload, apoptosis could be induced by other means (other cytokines) and the effect of IL-6 evaluated. Moreover, thorough investigation of the signalling pathways activated by IL-6 in purified FACS sorted α-cells and β-cells will elucidate whether IL-6 activates similar pathways in the α-cell and β-cell. These studies will shed light on the overall physiological relevance of IL-6 actions in the pancreatic islet.

The present work has provided substantial evidence that the IL-6 receptor is expressed in the α-cell, however it is not as clear whether this receptor is present on β-cells *in vivo*. It will be exciting to clarify this issue using islets from mice with a targeted deletion of the gp130 in β-cells (underway), thereby eliminating the possibility of a direct effect of IL-6 on the β-cell. Experiments performed on islets form these animals will not provide an answer with respect to β-cell expression of the IL-6 receptor, however these experiments will enable a conclusive statement on whether the observed effects of IL-6 in the present study were due to indirect effects of IL-6. Another interesting question remaining to be answered is if shedding of the IL-6 receptor from the α-cell membrane occurs *in vitro*, and whether this soluble IL-6 receptor plays a role in IL-6 activation of the β-cell. One approach to further elucidate this would be to block the possibility of the sIL-6 receptor to form heterodimers with the membrane bound gp130 on the β-cell. Using soluble gp130 as a naturally occurring antagonist would be one approach.

The interpretation of the lack of functional β-cell compensation in the IL-6 knockout mice on HF diet has been hypothesized to be due to the absence of an α-cell signal to the β-cell in these mice. This interpretation reflects the obvious caveat of the present study, which lies in the use of a systemic ligand knockout. Hence, it will be interesting to further clarify the role of IL-6 in the regulation of the individual pancreatic islet cells using cell specific deletion of the gp130. αgp130KO and βgp130KO mice are currently being created by breeding of gp130 flox/flox mice (kindly donated by Dr. Werner Müller, University of Manschester; see Betz, UA et al with Müller 1998 J Exp Med) with glucagon-Cre (kindly donated by Dr. P. Herrera, University of Geneva) and RIP-Cre (Jackson Labs) transgenic mice leading to selective disruption of gp130 in the α-cell and β-cell respectively. The availability of the αgp130KO and βgp130KO will elucidate the precise role of IL-6 in the regulation of pancreatic islet function, turnover and glucose homeostasis, and furthermore enable clarification of the importance of α-cell to β-cell communication and vice versa.

A further short coming of the present study is that it has only provided evidence that IL-6 is necessary for a HF diet induced increase in α-cell mass. Whether elevated IL-6 is also sufficient for α-cell mass expansion, would require IL-6 infusion/injection into wild type mice and evaluation of α-cell mass after a given period of time. Further, to conclude that IL-6 is both necessary and sufficient for α-cell mass expansion in the presence of metabolic stress *in vivo*, IL-6 should be replaced in IL-6 KO mice on HF diet to reverse this phenotype. While interesting for future investigation, these studies are beyond the scope of the present thesis.

The recent observation that HF diet induces PC1/3 expression in α-cells in wild type mice provides the basis for yet another unexplored avenue of research. It will be interesting to further investigate the role of IL-6 in this context, and to elucidate the role of IL-6 in α-cell produced GLP-1 under conditions of metabolically induced stress. The complete absence of measurable GLP-1 in the IL-6 knockout mice furthermore provide sufficient rational for investigating whether IL-6 plays a role in GLP-1 production from the major source the intestinal L-cells.

Based on IL-6 having a role in liver regeneration, it would be interesting to further explore whether IL-6 also plays a role in regeneration of islet mass. Therefore, creating an environment that stimulates β-cell regeneration in the systemic IL-6 knockout, the αgp130KO, and the βgp130KO would be of great interest, and is possible to achieve by administration of STZ. Importantly, α-cell mass expansion occurs following STZ treatment by unknown mechanism, thus treating the IL-6 knockout mice and wild type mice with STZ in the presence of an IL-6 receptor antagonist could provide information whether IL-6 is necessary and sufficient for α-cell mass expansion in this model.

The availability of the αgp130KO and βgp130KO mice will also allow investigation of the role of IL-6 in exercise to be taken a step further, and possibly add knowledge to the concept of IL-6 being an "exercise factor" with a yet unknown role. The hypothesis to be investigated is whether IL-6 released from the working skeletal muscle during exercise is acting on the pancreatic α-cell to increase glucagon and thereby substrate availability by means of increased hepatic glucose output. Comparison of endurance capacity, glucagon levels, glucose rate of appearance in wild type and αgp130 KO would further clarify this potential role for IL-6.

The results obtained in the present study support the pleiotropic nature of IL-6, and are furthermore well illustrated by the fact that an IL-6 receptor antagonist has reached/is about to reach FDA approval for the treatment of Rheumatoid Arthritis (personal communication at the 2008 Keystone Symposia) at the same time as studies are ongoing aiming at using IL-6 as an anti-obesity agent (Febbraio, 2007) (Wallenius et al., 2003) (Jansson et al., 2003). Considering these two clinical approaches, one blocking and the other enhancing IL-6 actions in the context of the present thesis work is associated with some concern. One can envision that further increasing exogenous IL-6 will cause an expansion of α-cell mass under conditions where an expansion of α-cell mass is not required for the maintenance of β-cell function, and thus lead to the deterioration of glucose homeostasis, and partly blocking IL-6 actions may lead to β-cell dysfunction. This scenario captures the essence of physiology; too much and too little might be equally bad.

Thus, in summary, the work presented proposes that elevated IL-6 levels during obesity drive α-cell mass expansion and glucagon expression, an early event that may be required for functional β-cell compensation in response to HF diet induced insulin resistance. However, whereas increased IL-6 levels at an early stage seem to be beneficial, prolonged elevated IL-6 levels may lead to pathological glucagon secretion observed at onset and during progression of diabetes.

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